Development and validation of high-throughput real-time PCR systems for diagnosis and typing of pathogens in pigs

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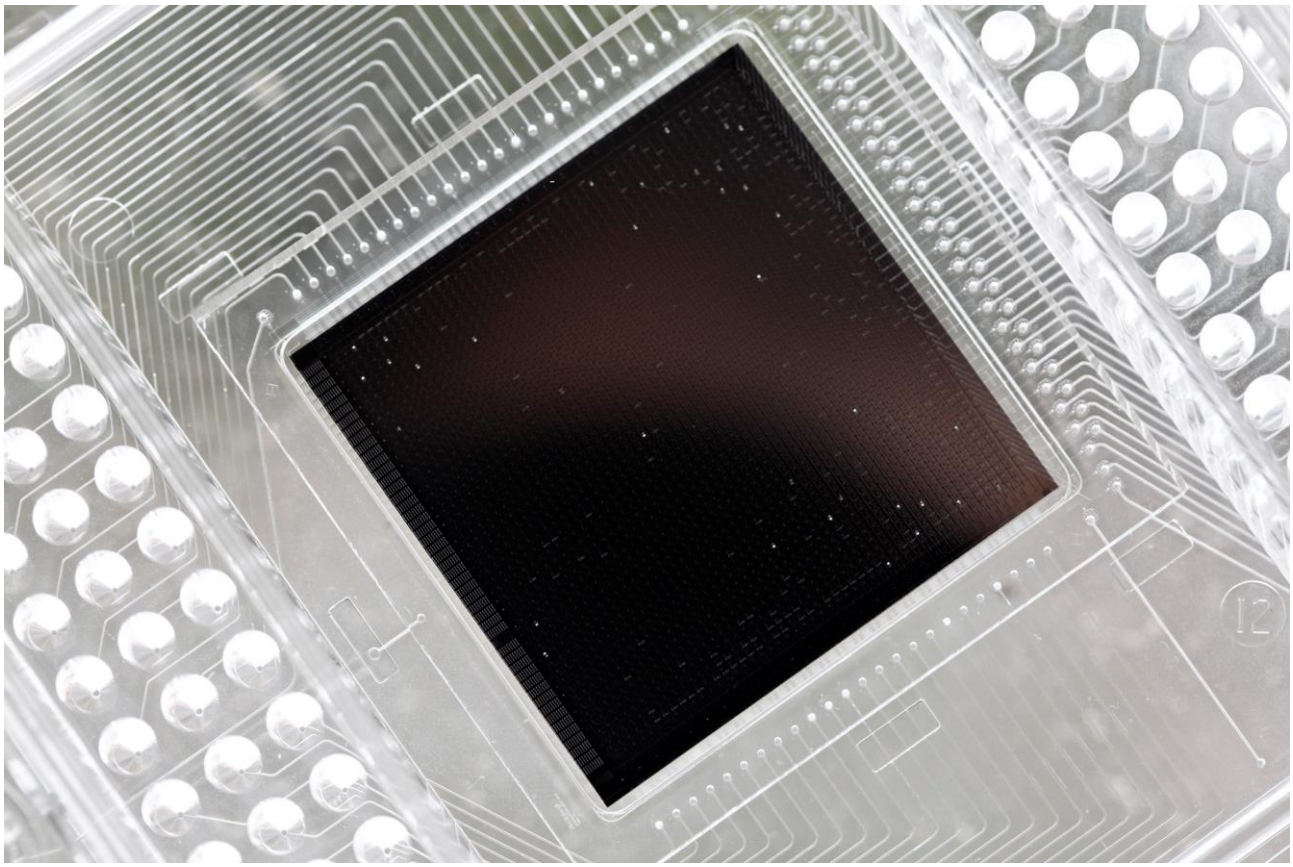

PhD thesis
Nicole Bakkegård Goecke
May 2018

DTU Vet
National Veterinary Institute

Development and validation of high-throughput real-time PCR systems for diagnosis and typing of pathogens in pigs

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Development and validation of high-throughput PCR systems for diagnosis and typing of pathogens in pigs

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Preface and acknowledgements

This PhD thesis is based upon work performed in the virology group at the Division for Diagnostics and Scientific Advice, National Veterinary Institute, Technical University of Denmark.

For me it has been a very interesting and educational time during the PhD project and there are a lot of people I would like to acknowledge for their help and guidance during this project. First of all, I would like to thank my supervisor Professor Lars Erik Larsen for giving me the opportunity to carry out this PhD project in his group and for his big support in all kinds of practical and academic subjects. I would also like to thank my co-supervisors Charlotte Kristiane Hjulsager and Kerstin Skovgaard for their guidance in different scientific and practical aspects of the PhD and for always taking their time to teach me new knowledge. A special thanks goes to Jesper Schak Krog for his every day help and for always taking the time to discuss the project with me. For technical assistance in the laboratory, I would thank Karin Tarp for her help and assistance with the high-throughput qPCR BioMark platform. Furthermore, I would like to thank the laboratory technicians Hue Thi Thanh Tran, Tine Skotte Hammer, Nina Dam Grønnegaard, Sari Mia Dose, Jonas Høgberg, Ivan Larsen and Sofie Hagedorn Nielsen for taking the time to answer questions and for the help with different tasks in the laboratory. A big thanks to my fellow PhD students Malene Rask Andersen, Pia Ryt-Hansen and Simon Welner for their good companionship and for all our talks about academic and everyday stuff. Thanks to the virology group including Katrine Fog Thomsen for being good colleagues and for many fun and enjoyable moments. Furthermore, thanks to Sven Erik Lind Jorsal, Lise Kirstine Kvisgaard, Helene Larsen, Linette Buxbom Nielsen and Klara Tølbøl Lauritsen for sharing of knowledge and thanks to Maja Kobberø and Thomas Kusk for their great effort with sampling all the samples used in manuscript III. Also big thanks to the National Veterinary Institute, Technical University of Denmark and the Danish Swine Levy Fund for funding the project, and thanks to the Otto Mønsteds fund and the Oticon fund for funding my participation in conferences.

I am grateful to Gaëlle Simon for giving me the opportunity to have a research stay at the ANSES laboratory in Ploufragan in France and for taking good care of me during my stay aboard. Furthermore, I would like to thank Stéphane Gorin for his kind assistance in the laboratory.

In the end I would like to thank my family and friends for their everlasting and invaluable support during the project. A special thanks goes to my friend Helene Lunde Robertsen, who has been an indispensable support.

Nicole Bakkegård Goecke, May 2018

List of abbreviations

cDNA	Complementary DNA
cRNA	Complementary RNA
C_q	Quantification cycle
DA	Dynamic array
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
HA	Hemagglutinin
HI	Haemagglutination inhibition
IAV	Influenza A virus
IFC	Integrated fluidic circuit
LNA	Locked nucleic acid
M	Matrix protein
NA	Neuraminidase
NEP	Nuclear export protein
NGS	Next generation sequencing
NP	Nucleoprotein
NS	Non-structural protein
nt	Nucleotides
NTC	Non-template control
PA	Acidic polymerase protein
PAR	Progressive atrophic rhinitis
PB1	Basic polymerase 1 protein
PB2	Basic polymerase 2 protein
PCMV	Porcine cytomegalovirus
PCR	Polymerase chain ceaction
PCV2	Porcine circovirus type 2
PCV3	Porcine circovirus type 3
POC	Point of care
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus

List of abbreviations

qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
swIAV	Swine influenza A virus
T_m	Melting temperature
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein

Summary

Millions of pigs are produced in Denmark each year, making it one of the largest pig producing countries in the world. Production of pigs can be associated with various challenges such as environmental and management issues and with the presence of pathogens. Respiratory and intestinal diseases caused by pathogens are of major importance to the pig production as it can result in reduced productivity, impaired animal welfare, increased mortality or morbidity. Vaccination and medical treatment are often initiated based on clinical observations and lacks support from laboratory investigations. This can lead to an overuse of medication and suboptimal vaccination programs. A reason why laboratory diagnostic analyses are often neglected is related to the high costs associated with the diagnostics and difficulties in interpretation of the results of random cross-sectional samples.

This PhD thesis consists of an introduction in which the project and relevant background is introduced. Here, the focus is on influenza A virus (IAV), especially in pigs, and on respiratory and enteric viral and bacterial pathogens, which all have an impact on the Danish pig production. Furthermore, the background contains a section describing the history of methodology used for diagnostic analyses with the greatest focus put on polymerase chain reaction (PCR). The results of the work performed during the PhD are presented in the form of three manuscripts. Finally, the three manuscripts and suggestions to further work are discussed in a broader context.

Manuscript I focus on the design and development of a high-throughput system to improve surveillance of swine influenza A virus (swIAV) and lower the costs of virus subtyping. For this, the high-throughput real-time PCR (qPCR) BioMark platform (Fluidigm) was used and optimized. To validate the platform, 12 different qPCR assays specific for various hemagglutinin and neuraminidase gene lineages relevant for swIAV and six different assays specific for the internal genes of the 2009 human pandemic strain (A(H1N1)pdm09) were tested. To test the sensitivity and specificity of the established high-throughput system, virus isolates and field samples, which had earlier been subtyped by full genome sequencing or multiplex reverse transcription qPCR (RT-qPCR), respectively, were used. Based on our findings, it was concluded that the high-throughput system is sensitive and specific and, thereby, the system provides a cost-effective alternative for subtyping of swIAVs.

In manuscript II, a high-throughput diagnostic system specific for 17 respiratory and enteric viral and bacterial pathogens was designed and established again using the high-throughput qPCR BioMark platform (Fluidigm). A total of 21 qPCR assays were validated and optimized to run under

the same reaction conditions. Furthermore, the sensitivity and specificity were assessed based on testing of known positive samples. The results revealed a performance of the diagnostic system similar to that of the qPCR analyses on the current qPCR platform. In addition, by using the high-throughput diagnostic system it is possible to offer diagnostic services with reduced costs and turnover time.

Manuscript III describes three monthly sampling of oral fluid and faecal sock samples together with observations of clinical signs in six nursery and four finisher herds in Denmark. The collected samples were analysed with the high-throughput diagnostic system described in manuscript II and the occurrence of significant correlation between presence of pathogen and clinical signs, such as coughing and diarrhoea, was investigated. The study revealed that swIAV, porcine circovirus type 2 (PCV2), porcine cytomegalovirus (PCMV), *Brachyspira pilosicoli*, *Lawsonia intracellularis*, *Escherichia coli* fimbria type F4 and F18 were prevalent in several of the herds. Furthermore, for some of the pathogens a significant correlation was observed between their detection and the presence of coughing and/or diarrhoeic events. The study further showed that the use of the high-throughput diagnostic system for continuous monitoring of pathogens provides a broader knowledge on the distribution of pathogens in a given herd. This information can be useful for veterinary consultancy as it creates a more objective basis for intervention, including treatment regimens and vaccination strategies.

Based on the studies described in manuscripts I, II and III, it was concluded that the use of the high-throughput qPCR BioMark platform provides a new and innovative way of carrying out diagnostic analyses. Changing to this cheaper platform will favour the use of laboratory analyses in the pig industry and provide a more objective basis for the selection of preventive measures to the benefit of animal health, animal welfare, production economics and food safety. The high-throughput system is presently under establishment in the Diagnostic Centre at the National Veterinary Institute, Technical University of Denmark and it will soon be offered to costumers as a diagnostic service.

Resumé (summary in Danish)

I Danmark produceres årligt millioner af grise, hvilket gør det til et af verdens største svineproduktionslande. Produktion af grise kan være forbundet med forskellige udfordringer såsom miljø- og håndteringsproblemer og tilstedeværelsen af patogener. Respiratoriske- og tarmsygdomme forårsaget af patogener har stor betydning for svineproduktionen idet de kan medføre nedsat produktivitet, forringet dyrevelfærd, øget dødelighed og sygelighed. Vaccination og medicinsk behandling påbegyndes ofte baseret på kliniske observationer og uden støtte fra laboratorieundersøgelser. Dette kan føre til overforbrug af medicin og suboptimale vaccinationsprogrammer. En af årsagerne til at laboratoriediagnostiske analyser ofte forsømmes, er relateret til de høje omkostninger forbundet med diagnostik og vanskeligheder forbundet med fortolkning af resultater fra tilfældige tværsnitsprøver.

Denne PhD afhandling består af en introduktion, hvor projektet og den relevante baggrund introduceres. Her er det primære fokus lagt på influenza A virus (IAV), især hos svin, og på respiratoriske og enteriske virale og bakterielle patogener, som alle har indflydelse på den danske svineproduktion. Desuden indeholder baggrunden et afsnit omhandlende de typiske metoder anvendt til diagnostiske analyser med et særligt fokus på polymerasekædereaktion (PCR). Resultaterne af det arbejde, der er udført under PhD-studiet, præsenteres i form af tre manuskripter. Endelig diskuteres resultaterne af de tre manuskripter og forslag til videre arbejde i en bredere sammenhæng.

Manuskript I fokuserer på design og udvikling af et high-throughput system til forbedret overvågning af svine influenza A virus (swIAV) og reducering af omkostningerne ved virus subtyping. Til dette blev high-throughput real-time PCR (qPCR) BioMark platformen (Fluidigm) brugt og optimeret. Til validering af platformen blev 12 forskellige qPCR assays med specificitet for forskellige hemagglutinin- og neuraminidase gener, der er relevante for swIAV, og seks forskellige assays, der var specifikke for de interne gener af den 2009 humane pandemisk stamme (A(H1N1) pdm09), testet. For at teste følsomheden og specificiteten af det etablerede high-throughput system anvendtes virusisolater og feltprøver som tidligere var subtypet med henholdsvis fuld genom-sekventering eller multiplex revers transkription qPCR (RT-qPCR). Baseret på vores resultater kunne det konkluderes, at high-throughput systemet er følsomt og specifikt, hvilket gør dette system til et omkostningseffektivt alternativ til subtyping af swIAV.

Resumé

I manuskript II beskrives etableringen af et high-throughput diagnostisk system specifikt for 17 respiratoriske og enteriske virale og bakterielle patogener igen ved anvendelse af high-throughput qPCR BioMark platformen (Fluidigm). I dette system blev i alt 21 qPCR assays valideret og optimeret til at køre under de samme reaktionsbetingelser. Endvidere blev følsomheden og specificiteten vurderet baseret på testning af kendte positive prøver. Resultaterne afslørede at præstationen af det diagnostiske system svarer til den nuværende qPCR platform. Dermed er det muligt at tilbyde diagnostiske tjenester med reducerede omkostninger og svartid ved at anvende det nyudviklede high-throughput diagnostiske system.

Manuskript III beskriver tre månedlige prøveudtagninger af spyt og fækale sokkeprøver sammen med observationer af kliniske tegn i seks fravænnings- og fire slagtesvinsbesætninger i Danmark. De indsamlede prøver blev analyseret med systemet beskrevet i manuskript II og forekomsten af patogener og korrelation mellem tilstedeværelse af patogener og kliniske tegn, såsom hoste og diarré, blev undersøgt. Undersøgelsen viste, at swIAV, porcine circovirus type 2 (PCV2), porcin cytomegalovirus (PCMV), *Brachyspira pilosicoli*, *Lawsonia intracellularis*, *Escherichia coli* fimbrie type F4 og F18 var fremherskende i flere af besætningerne. Endvidere blev der for nogle af patogenerne observeret en signifikant korrelation mellem deres påvisning og tilstedeværelsen af hoste- og/eller diarré. Undersøgelsen viste endvidere at brugen af et high-throughput diagnostisk system til vedvarende overvågning af patogener giver en bredere viden om fordelingen af patogener i en given besætning. Disse oplysninger kan være nyttige i veterinærrådgivning da det skaber et mere objektivt grundlag for intervention, herunder behandlingsregimer og vaccinationsstrategier.

Baseret på de undersøgelser, der er beskrevet i manuskripterne I, II og III, blev det konkluderet, at brugen af high-throughput qPCR BioMark platformen giver en ny og innovativ måde at udføre diagnostiske analyser på. Ved at skifte til denne platform er det muligt for brugerne at reducere omkostninger og tid brugt på analyse. Dette vil favorisere brugen af laboratorieanalyser i svineindustrien og give et mere objektivt grundlag for udvælgelse af forebyggende foranstaltninger til gavn for dyresundhed, dyrevelfærd, produktionsøkonomi og fødevaresikkerhed. High-throughput systemet er i øjeblikket under etablering i det Diagnostiske Center hos Veterinærinstituttet, Danmarks Tekniske Universitet, og det vil snart blive tilbudt til forbrugerne som en diagnostisk service.

1 Introduction and aims

In 2015, the National Veterinary Institute, Technical University of Denmark, started a project in collaboration with SEGES and University of Copenhagen with the title "Improved surveillance of health and production in pigs". The cooperation aimed to improve the health monitoring programs in pig herds to ensure more efficient and rapid detection of infectious diseases in the early stage of disease. In addition, it should improve identification of the infections playing the most significant role in the herds. A part of the project was to develop a diagnostic system suitable for analysis of a high number of samples and for screening for several different pathogens simultaneously. As a part of this project, the Danish Swine Leary Foundation supported a PhD project at the National Veterinary Institute, Technical University of Denmark to establish a laboratory diagnostic system that could fulfil these criteria.

Thus, the **main aim** of this PhD project was the development of high-throughput diagnostic systems for detection and typing of veterinary pathogens (viruses and bacteria) with major importance to the health and welfare of Danish swine.

The main activities of the PhD comprise three projects with the following focuses:

1. Establishment, optimization and validation of a high-throughput system for rapid and specific subtyping of swine influenza A viruses (swIAVs) (**manuscript I**)
2. Establishment, optimization and validation of a high-throughput diagnostic system specific for the most relevant Danish porcine viral and bacterial pathogens (**manuscript II**)
3. Validation of the high-throughput diagnostic system (manuscript II) on field samples from nursery and finisher pigs collected as a part of a prospective study in ten Danish pig herds. Here, the high-throughput diagnostic system was used to analyse the connection between detection of pathogen(s) and the observed clinical signs of diseases (**manuscript III**)

1.1 Outline of the PhD thesis

The **background** section contains an overall introduction to influenza A virus (IAV) in pigs, and to respiratory and enteric viral and bacterial pathogens with impact on the Danish pig production. Furthermore, the methodology used for the diagnostic analyses with the greatest focus put on polymerase chain reaction (PCR) is described. The **own studies** section contains the three manuscripts of the PhD project. The **discussion and perspectives** section contains a general

discussion of the PhD project and suggestions to further work. Lastly, the PhD thesis is completed with a **conclusion** section in which the main findings are highlighted.

2 Background

2.1 Influenza A virus

2.1.1 Taxonomy and structure

The genus *Influenza virus A* belongs to the family Orthomyxoviridae, which also comprises the genera *Influenza virus B, Influenza virus C, Thogotovirus, Isavirus* and the newly described *Influenza virus D* (Hause et al., 2014; Webster et al., 1992). The natural reservoir of influenza A viruses (IAVs) are aquatic birds, however, the virus has been isolated from a wide range of species including pigs, seals, humans, cats, dogs and horses (Webster et al., 1992). The clinical signs of IAV include acute onset of disease, fever, anorexia, coughing, nasal discharge, sneezing, exhaustion and apathy (Zell et al., 2013). Influenza B viruses have been isolated from humans and seals and the clinical symptoms caused by these are similar to those of IAV, but in a milder form. Influenza C viruses have been isolated from humans and swine and generally cause only mild disease in the upper respiratory tract (Baigent and McCauley, 2003; Osterhaus et al., 2000). The newly discovered influenza D viruses have so far been isolated from pigs and cattle affected by respiratory disease (Chiapponi et al., 2016; Ducatez et al., 2015; Hause et al., 2014).

IAV is an enveloped single-stranded, negative sense RNA virus with a segmented genome of approximately 13.6 kb. In general, the IAV particles are pleomorphic and roughly spherical with a size of 80-120 nm in diameter, however, some are filamentous with a size of 1 µm or longer (Fujiyoshi et al., 1994; Webster et al., 1992). The genome of IAV consists of eight gene segments (Figure 1), which are coding for the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), basic polymerase 1 protein (PB1), basic polymerase 2 protein (PB2), acidic polymerase protein (PA), nucleoprotein (NP), matrix proteins (M1 and M2) and the non-structural proteins (NS1 and NS2, also called nuclear export protein (NEP)). The size of the gene segments range between 890-2341 nucleotides (230-759 amino acids). Some of the segments encode a single protein, whereas other segments encode for several different proteins, where the function for some of the detected proteins have not been fully elucidated yet (Bouvier and Palese, 2008; te Velthuis and Fodor, 2016). The viral envelope consists of a host-derived lipid bilayer in which the transmembrane proteins HA, NA and M2 are embedded. While HA and NA are anchored in the lipid bilayer, the cholesterol-binding protein M2 is not tightly associated with the lipid bilayer.

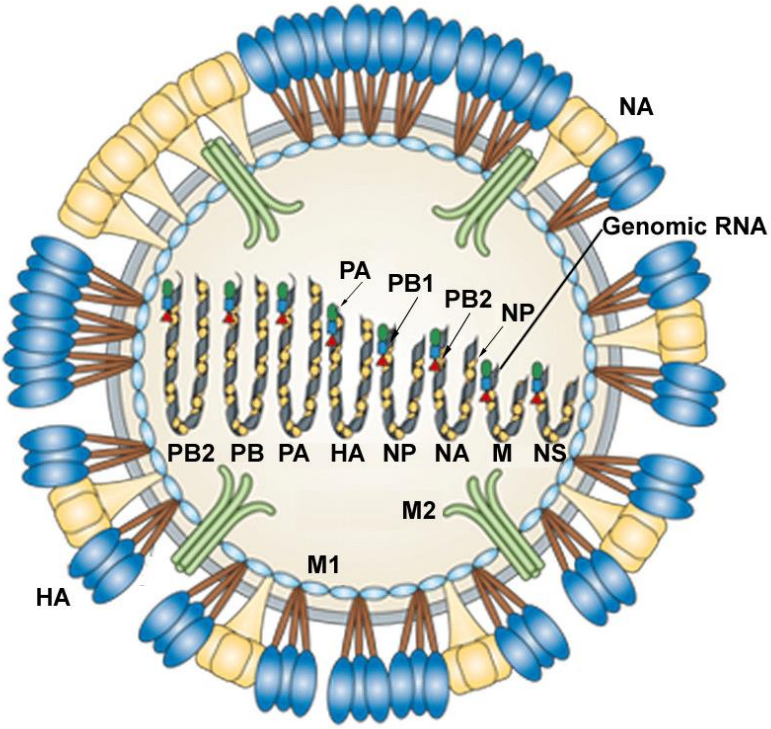

Figure 1 Schematic representation of Influenza A virus. The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) and the matrix 2 (M2) protein are located in the viral envelope. The matrix 1 (M1) protein is anchored inside the viral envelope underneath the lipid membrane. In the virion core, each RNA segments are complexed with the polymerase complex (PA, PB1 and PB2) and the nucleoprotein (NP) to form viral ribonucleoproteins (vRNPs) (Scolari et al., 2009).

The major envelope protein HA is a homotrimer responsible for receptor binding and membrane fusion and consists of individual monomers composed of the two polypeptide chains HA1 and HA2. The second most abundant envelope protein NA is a homotetramer, which function is to cleave receptor binding (sialic acid linkages) and release the viral progeny. M2, the least abundant envelope protein, is a homotetramer functioning as a ion channel (Nayak et al., 2009; Schroeder et al., 2005; Webster et al., 1992). The M1 protein is anchored inside the viral envelope underneath the lipid membrane where it forms a shell surrounding the virion core. Inside the virion core, each viral RNA (vRNA) segment is folded into a rod-shaped double-helix viral ribonucleoprotein complex (vRNP), where the vRNA segments are bound to the NP protein and to the heterotrimeric viral polymerase complex consisting of PB1, PB2 and PA. The vRNPs play an important role in the virus infection cycle (Cheung and Poon, 2007; Webster et al., 1992; Zheng and Tao, 2013).

2.1.2 The replication cycle of influenza A virus

The first stage in viral infection caused by IAVs is the recognition and binding of HA to α2.3- or α2.6-linked sialic acid containing receptors on the host cell surface. IAVs from different host species show different receptor binding preferences; in general, human and swine IAVs favour binding to α2.6-linked sialic acid, whereas avian IAVs shows preference for the α2.3-linked sialic acid. The sialic acid binding specificity of HA is one of the major determinants for controlling viral tropism and host specificity (Imai and Kawaoka, 2012; Shinya et al., 2006; Trebbien et al., 2011). Upon cell attachment, IAV enters the cell via receptor-mediated endocytosis (Figure 2).

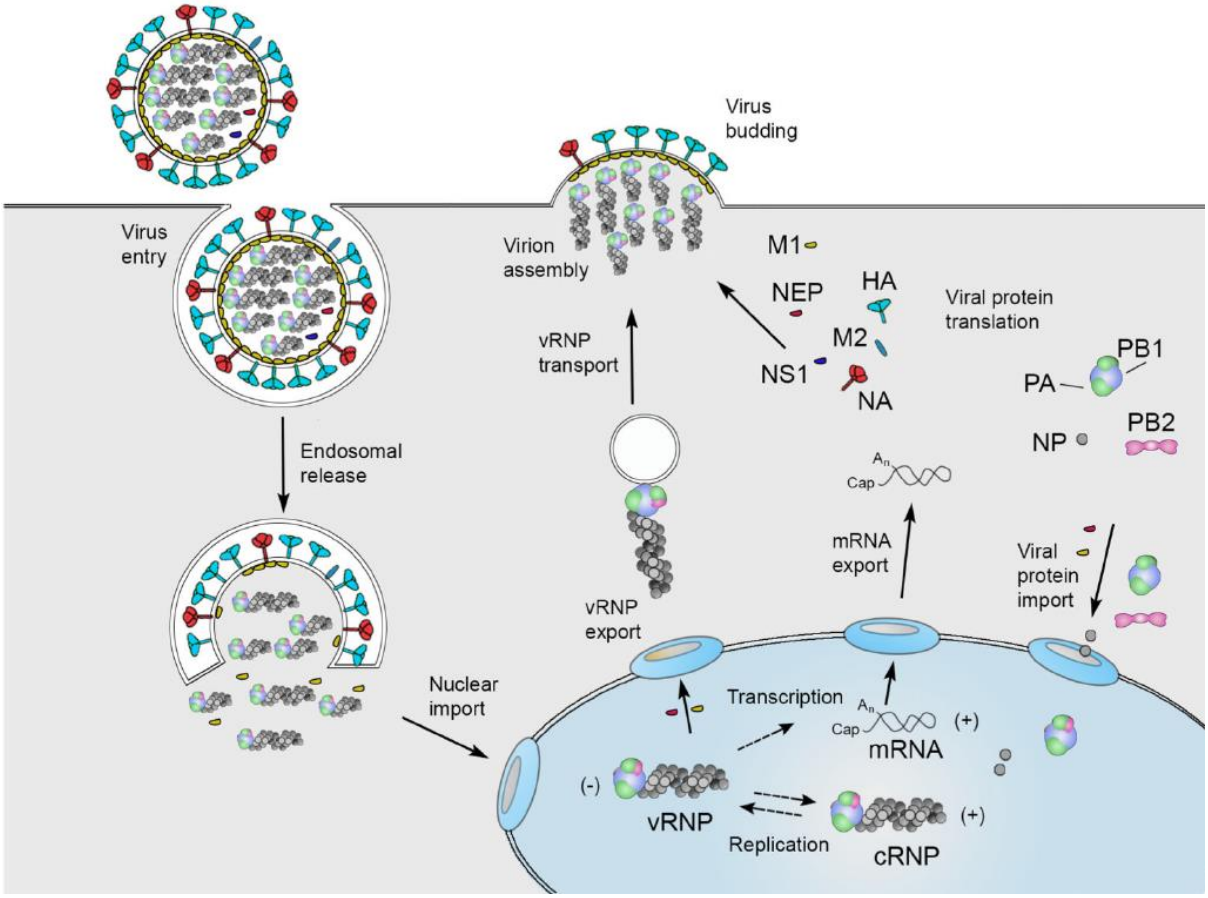

Figure 2 Schematic representation of the influenza A virus replication cycle. Influenza A virus binds to sialic acid containing receptors on the host cell surface and the virus enters the cell by receptor-mediated endocytosis. The acidic environment in the endosome facilitates the release of the vRNPs into the host cell cytoplasm. The vRNPs is then transported into the nucleus where transcription and replication takes place. Polyadenylated and capped viral mRNA are exported to the cytoplasm for translation into viral proteins by the host translational machinery. Some of the newly synthesized viral proteins enter the nucleus to be included in new vRNPs. The newly formed vRNPs are exported from the nucleus and transported to the site of assembly at the host cell membrane, where new virions are packaged and released (te Velthuis et al. 2016).

The low pH of the endosome triggers a conformational change of the HA monomers (HA0), which is cleaved into HA1 and HA2 by trypsin-like enzymes (Lazarowitz et al., 1973). The cleavage of HA results in generation of the fusion peptide at the N-terminus of HA2, which mediates the fusion of the viral membrane with the endosomal membrane and ultimately the release of the vRNPs into the host cell cytoplasm. The vRNPs are then transported into the host cell nucleus through nuclear pores by means of virus proteins' nuclear localization signals for transcription and replication of vRNA (Martin and Helenius, 1992). Inside the nucleus, the viral RNA-dependent RNA polymerase (RdRp – comprised of PB1, PB2, PA) uses the negative-sense vRNA as a template for synthesis of messenger RNA (mRNA), which then acts as template for viral protein synthesis, and for synthesis of the positive-sense complementary RNA (cRNA) intermediate, which serves as a template for production of new negative-sense genomic vRNA (Bouvier and Palese, 2008). Transcription is activated in the nucleus and is initiated by an event named "cap-snatching", in which the PB2 subunit binds to the 5' cap end of the host pre-mRNA, which are then cleaved after 10–15 nucleotides by the endonucleolytic activity of the PA subunit. The short capped leader sequence is subsequently used as a viral primer for extension, which is carried out by the PB1 subunit, using vRNA as a template, generating capped viral mRNAs (Dias et al., 2009; Plotch et al., 1981; York and Fodor, 2013). The presence of a short sequence of five to seven uracil (U) residues in the vRNA results in the transcription of viral mRNA with a string of adenosine residues that form a poly(A) tail. Polyadenylated and capped viral mRNA are then exported to the cytoplasm for translation into viral proteins by the host translational machinery. Furthermore, vRNA is also used as template for synthesis of positive-sense cRNAs, which are replication intermediates responsible for directing the synthesis of new negative-sense vRNAs. Newly translated NP, PB1, PB2 and PA are imported back to the nucleus, where newly synthesised vRNAs are encapsidated by free NP leading to the formation of new vRNPs, which are then exported from the nucleus and transported across the cytoplasm to the cell membrane where virus assembly takes place. Nuclear export of nascent mRNAs and vRNPs into the cytoplasm is facilitated by M1 and NEP (Cros and Palese, 2003; Zheng and Tao, 2013). Besides permitting nucleus export, M1 is also involved in transcriptional downregulation (Perez and Donis, 1998). The newly translated envelope proteins HA, NA and M2 are transported to the cell membrane after passing through the host cell secretory pathway for post-translational modification. At the host cell membrane, these proteins are incorporated into the lipid bilayer and together with an accumulation of the M1 protein at the cytoplasmic site of the cell membrane initiates the budding process. In the budding virus particle,

vRNPs are packed together with the mentioned proteins. The newly formed virion remains bound to the host cell membrane until the sialic acid containing receptor is cleaved by the enzymatic activity of NA, which entails release from the host cell. For the new IAV to be infectious, successful assembling and packing of virion are required (Bouvier and Palese, 2008; Kuiken et al., 2006).

2.1.3 Evolution of influenza A virus

The genetic diversity of IAVs is enormous and the antigenic evolution of viruses is mainly mediated through mutation of the virus itself, called antigenic drift, and through exchange of gene segments, referred to as antigenic shift or reassortment. The antigenic drift is the gradual evolution of IAVs caused by frequent mutations, which occur due to lack of proofreading capacity of the viral RNA-polymerase (viral quasispecies). Selection pressure on the virus ensures that a small subset of the new virus variants contains some advantage over the parent and, hence, only the positive selected variants will survive. Mutations that cause changes in the antibody binding site will inhibit the efficiency of the host antibodies with specificity towards previously circulating strains and this will allow the virus to spread more rapidly among the population (Carrat and Flahault, 2007; Zambon, 1999).

Antigenic shift or reassortment occurs when a host cell is simultaneously infected by more than one IAV strain (Figure 3). In such a case, the gene segments from the different IAVs can be interchanged during the virion packaging and a new IAV variant is produced. The new reassorted strain can end up containing a genetic composition, which is entirely unknown for the population, which in the worst case can cause a new IAV pandemic (Zambon, 1999).

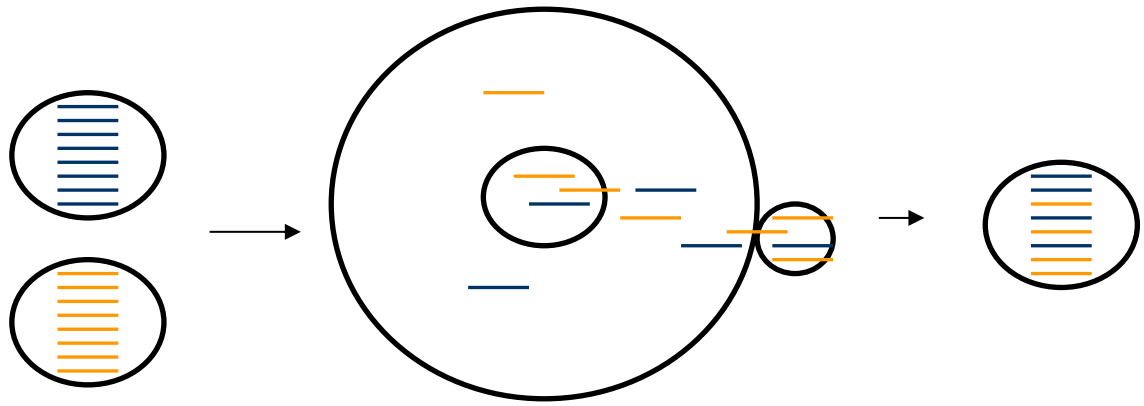

Figure 3 Schematic drawing illustrating antigenic shift/reassortment. Two influenza A viruses with different gene segments (blue and orange) are infecting the same cell. The gene segments are interchanged during the virion packaging and a new influenza A virus variant is produced. The drawing is made by Ramona Trebbien.

2.1.4 Classification of influenza A virus

Classification of IAVs into subtypes are based on the surface glycoproteins HA and NA. Currently, 16 different HA (H1-H16) and nine different NA (N1-N9) subtypes have been described and isolated from aquatic birds. Recently, the subtypes H17N10 and H18N11 have been discovered, but they have so far only been isolated from bats (Fouchier et al., 2005; Wu et al., 2014).

2.1.5 Influenza A virus in European pigs

Influenza A virus in swine (swIAV) was first observed in 1918, which coincided with the human influenza pandemic "the Spanish flu" which resulted in the death of millions of people worldwide. It was not before 1930 that the virus was isolated and identified (Shope, 1931). Later, the virus was classified as an H1N1 subtype and named the "classical swine influenza", which is used today for H1N1 subtypes with similarity to the original strain (Brown, 2000; Reid et al., 1999). After the discovery of the classical swine H1N1 virus, the virus continued to circulate in the United States, but disappeared from Europe until 1976 when the virus reappeared in Italy and spread to other European countries (Brown, 2000; Nardelli et al., 1978). Today, the dominant H1N1 subtype in European pigs is of avian origin, referred to as avian-like H1N1, which was introduced from waterfowls to pigs around 1979 (Pensaert et al., 1981). The avian-like H1N1 is genetically and antigenically distinct from the classical swine H1N1. The classical swine H1N1 has been replaced by the avian-like H1N1 in Europe, where it is now one of the most predominant subtype (Brown, 2013; Simon et al., 2014).

Following the 1968 human pandemic, referred to as the "Hong Kong flu", an H3N2 virus of human origin was identified in pigs. In the 1970s, the virus was transmitted to and got established in pigs, however, without causing any clinical signs of disease (Miwa et al., 1987; Ottis et al., 1982). Around 1984, a reassorted H3N2, which induce typical swine influenza symptoms, was discovered. This virus was a result of a reassortment event between the human-like H3N2 and the avian-like H1N1, in which the HA and NA genes were of human origin and the six other gene segments of avian descent (Castrucci et al., 1993). This porcine-like H3N2 lineage is now the dominant genotype of the H3N2 subtype in European pigs (Simon et al., 2014).

In the late 1980s, an H1N2 virus with HA origins from the classical swine H1N1 and NA from the porcine-like H3N2 was isolated from pigs in France (Gourreau et al., 1994). However, although the H1N2 virus was associated with clinical disease it did not appear to spread widely in Europe. In 1994, another H1N2 reassortant was isolated for the first time in Great Britain. In this case, the HA

gene was derived from a human seasonal H1N1 virus, which was circulating in humans in the 1980s, while the NA gene originated from the porcine-like H3N2 and the internal genes from the avian-like H1N1 (Brown et al., 1995, 1998). This human-like reassortaed swine H1N2 is the predominant virus within this subtype in Europe, but it has never been detected in Denmark. Furthermore, in 2003 a new reassortant H1N2, containing the HA gene from the avian-like H1N1 and the NA gene from the porcine-like H3N2, was found in Denmark (Trebbien et al., 2013). This avian-like H1N2 virus became established in several European countries including Denmark, where it is now one of the most prevalent subtypes (Simon et al., 2014).

Following the human pandemic in 2009, caused by a novel H1N1 virus, a fourth IAV lineage entered the global pig population. The human pandemic H1N1 strain (A(H1N1)pdm09) is now enzootic in pigs globally and this virus contains a unique gene constellation not previously described or reported. Six of the gene segments (PB2, PB1, PA, HA, NP, NS) show close homology to the North American triple-reassortant swIAV, whereas the last two segments (NA, M) show closest homology to the Eurasian avian-like H1N1 linage (Garten et al., 2009; Smith et al., 2009). The A(H1N1)pdm09 virus has been established in several European countries including Denmark, where it is one of the most prevalent subtypes (Simon et al., 2014). Furthermore, reassortants between the predominant enzootic swIAVs and the A(H1N1)pdm09 have been observed (Starick et al., 2011; Watson et al., 2015) and together with spillover of seasonal human H3 and human N2 segments, which have been observed in Danish pigs (Breum et al., 2013; Krog et al., 2017), these reassortants can carry zoonotic and even pandemic potential.

2.2 Respiratory pig pathogens

Respiratory diseases are one of the major diseases in pigs worldwide and are of great economic importance due to clinical and subclinical diseases, which can influence productivity, reduce animal welfare and can have high treatment cost (Hansen et al., 2010). Respiratory diseases can be caused by a wide range of pathogens, and in most cases is the result of a multifactorial problem including several pathogens (Palzer et al., 2008). Often a primary infection can pave the way for a secondary infection, which can complicate the course of disease and thereby delay the recovery and in the worst case lead to death. Pathogens causing respiratory diseases vary significantly between countries, regions, production sites and herds (Opriessnig et al., 2011). Selected respiratory disease-causing pathogens with high impact in the Danish pig production will be described in the following sections with focus on pathogenesis and clinical signs.

2.2.1 Pathogenesis and clinical signs of virus infections

2.2.1.1 Porcine cytomegalovirus

Porcine cytomegalovirus (PCMV) is an enveloped virus with a double stranded linear DNA genome and belongs to the family *Herpesviridae*, the subfamily *Betaherpesvirinae* and the genus *Cytomegalovirus* (Roizmann et al., 1992). PCMV is enzootic in pig populations throughout the world and infection can occur in all age groups. The virus can persist latently within cells and can be activated during stress or immunosuppression (Mueller and Fishman, 2004). The major sites of infection are the nasal turbinates and the upper respiratory tract. Infection with PCMV can lead to foetal and piglet death, inclusion body rhinitis, pneumonia, dysplasia and poor weight gain in susceptible herds, while in herds where management conditions tend to be good the virus may be endemic without causing any clinical disease (Hamel et al., 1999; Liu et al., 2013).

2.2.1.2 Porcine circovirus type 2 and porcine circovirus type 3

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, circular single-stranded DNA virus, belonging to the family *Cirocoviridae* and the genus *Circovirus* (Meehan et al., 1998). PCV2 is widespread in pig herds throughout the world and four genotypes termed a, b, c and d have been described for PCV2 (Dupont et al., 2008; Segalés et al., 2008; Xiao et al., 2015). PCV2 is associated with a number of disease syndromes, such as post-weaning multisystemic wasting syndrome (PMWS), in which PCV2 is considered to be the causative agent. PMWS affects pigs predominantly between five and 15 weeks of age and the syndrome is characterized by wasting, dyspnoea, paleness of the skin, lesions in lymphoid tissues, enlarged lymph nodes, diarrhoea and increased mortality (Allan and Ellis, 2000; Baekbo et al., 2012; Segalés and Domingo, 2002). Not all pigs infected by PCV2 will develop PMWS, since it is a multifactorial disease in which other factors in addition to PCV2 are needed to trigger clinical disease (Segalés et al., 2005). Besides PMWS, PCV2 has also been linked to enteritis, pneumonia, porcine dermatitis and nephropathy syndrome (PDNS) and to reproductive problems (Allan et al., 2000; Allan and Ellis, 2000; Sanchez et al., 2001). Replication of PCV2 in lymphoid tissues can occurs in B and T lymphocytes, monocytes and macrophages and in pigs affected with PMWS lymphocyte depletion and infiltration of monocytes in lymphoid tissues have been observed (Chianini et al., 2003; Sanchez et al., 2004; Yu et al., 2007).

A novel type of porcine circovirus, named porcine circovirus type 3 (PCV3), was described for the first time in November 2016 in USA (Phan et al., 2016) and has subsequently also been detected in Asia and Europe including Denmark (Franzo et al., 2018; Ku et al., 2017; Stadejek et al., 2017). PCV3 is genetically quite different from PCV2, since the two viruses only share 50% similarity. Currently, the knowledge about PCV3 is based on only a few studies, thus future analyses are needed to clarify the prevalence and disease history of this novel virus.

2.2.1.3 Porcine parvovirus

Porcine parvovirus (PPV) belongs to the family *Parvoviridae* and is a small non-enveloped virus with a linear genome of single-stranded DNA. PPV, which occurs as a single serotype, is found in pig herds throughout the world, where it is enzootic in many of the herds, although the impact of disease has been substantially reduced by vaccination. The virus is an important cause of reproductive failure in pigs and infection with PPV can lead to stillbirth, mummification, embryonic death and infertility (SMEDI). The virus can replicate in different tissues and organs, including lymph nodes, tonsils, thymus, salivary glands, spleen and lungs, and, furthermore, it replicates well in blood lymphocytes, monocytes and macrophages. PPV infection of older pigs often causes only mild or subclinical disease, however, in rare cases the virus has also been associated with respiratory, vesicular and systemic disease of neonates. The major impact of PPV is infection of pregnant gilts and sows and the stage of gestation at which infection occurs determines the particular clinical signs observed. Infection of embryos in the first weeks of life results in death and resorption, while infection later in the gestation, but before day 70, results in dead of the foetuses, which can become mummified. Foetuses infected after 70 days of gestation are usually less affected due to development of an immune response (immune competence of pig foetuses starts at 60–70 days) (Huysman et al., 1992; Mengeling et al., 2000; Woods and McDowell, 2009).

2.2.1.4 Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, enveloped, single-stranded RNA virus, which belongs to the family *Arteriviridae* and the genus *Rodartevirus* (Conzelmann et al., 1993; Kuhn et al., 2016; Meulenberg et al., 1993). The syndrome is characterized by reproductive failure in sows and respiratory disease in piglets and the virus is enzootic in pig herds throughout the world (Rossow, 1998). Two genotypes of PRRSV exists and genomic sequence analysis shows that there are only around 63 % nucleotide identity between

viruses isolated in Europe and in North America (Allende et al., 1999). Today, the viruses previously classified as European type are now classified as Type 1 and those previously classified as North America type as Type 2. Both types are distributed worldwide (Shi et al., 2010). Infection with PRRSV can occur through different routes, but the most frequent route is the respiratory route, where PRRSV targets and replicates in macrophages in the alveolar and lymphoid tissue (VanReeth, 1997). Clinical signs induced by a PRRSV infection vary according to the age and pregnancy status of the infected pig (Rossow, 1998). PRRSV infection of a breeding herd can leads to reproductive failure, which can result in abortion, early farrowing, an increase in the number of stillborns, mummified foetuses and weak neonatal piglets (Figure 4). Furthermore, PRRSV infection in weaned and growing pigs is characterized by anorexia, fever, lethargy and pneumonia, while respiratory distress and preweaning mortality are features of the disease in neonatal pigs. The clinical signs observed depend on the level of virulence of the infecting strain (Rossow, 1998; Zimmerman et al., 1997).

Figure 4 Mummified foetuses. Photo by Lise K. Kvisgaard.

2.2.1.5 Swine influenza A virus

swIAV is a respiratory infection caused by multiple subtypes of IAV, of which the predominant subtypes are H1N1, H1N2 and H3N2 and these viruses are enzootic in pigs worldwide. SwIAV infection starts in the upper respiratory tract by infection of the nasal, tracheal, and bronchial epithelium with eventually spread to the lung. The infection can be very acute, affecting most of the respiratory tract within only a few hours. Nasal virus shedding and virus replication in the lungs are short-lasting and limited to the first six or seven days after infection (Brown et al., 1993; De Vleeschauwer et al., 2009). The main symptoms of swine influenza are sudden onset of the disease, fever, lack of appetite, coughing, sneezing, nasal discharge, dyspnoea, exhaustion and abortion. The

severity of disease ranges from subclinical to acute and this is influenced by the infecting virus strain and the pig's immunity. The morbidity within an affected herd is often high, while the mortality is usually low but it depends on the virus strain and other factors such as mixed infections and age (Zell et al., 2013).

2.2.2 Pathogenesis and clinical signs of bacterial infections

2.2.2.1 *Actinobacillus pleuropneumoniae*

Actinobacillus pleuropneumoniae is a Gram-negative bacterium belonging to the *Pasteurellaceae* family and the genus *Actinobacillus*. *A. pleuropneumoniae* is classified into 18 serotypes, in which serotype 17 and 18 are currently recognized (Bossé et al., 2002; Gottschalk, 2015; Sárközi et al., 2015). All serotypes can cause disease, but difference in virulence exists and the prevalence of the different serotypes differs across the world. *A. pleuropneumoniae* is the causative agent of porcine pleuropneumonia and the disease is distributed in pig worldwide, affecting pigs in all ages (Chiers et al., 2010). The lower respiratory tract is the site where *A. pleuropneumoniae* causes tissue damage leading to clinical disease. Clinical signs of acute disease are anorexia, coughing, depression, dyspnoea, fever and in worst case the disease can lead to death. However, *A. pleuropneumoniae* can also be present in a subclinical form, in which the bacteria resides in the respiratory tract and tonsillar tissue of carrier pigs (Chiers et al., 2002). *A. pleuropneumoniae* binds to mucus, proteins and cells of the lower respiratory tract and several virulence factors like fimbriae and lipopolysaccharides may play a role in its adhesion (Belanger et al., 1990; Bertram, 1990). The bacterium produces four toxins designated ApxI-IV, which belong to the pore-forming RTX toxin family (Frey et al., 1993; Schaller et al., 1999). These toxins are the most significant virulence factors produced by the *A. pleuropneumoniae* and together they play an important role during the pathogenesis of the infection (Chiers et al., 2010).

2.2.2.2 *Bordetella bronchiseptica*

Bordetella bronchiseptica is a Gram-negative bacterium belonging to the family *Alcaligenaceae* and the genus *Bordetella*. *B. bronchiseptica* is widely distributed in pig herds throughout the world and is one of the microorganisms, associated with the complex aetiology of atrophic rhinitis (Collings and Rutter, 1985). In atrophic rhinitis cases where *B. bronchiseptica* is alone, only a mild form of disease will occur, while in herds where both *B. bronchiseptica* and *Pasteurella multocida* are present severe atrophic rhinitis can occur, which can lead to severe distortion and atrophy of the

turbinate bones in the nasal terminus. Clinical signs observed by *B. bronchiseptica* infections are fleas from eyes and nose and sneezing (Chanter et al., 1989; Giles et al., 1980). *B. bronchiseptica* binds to the mucous membranes in the upper respiratory tract and several virulence factors like adhesions and toxins may play a role in the infection (Goodnow, 1980).

2.2.2.3 *Haemophilus parasuis*

Haemophilus parasuis is a Gram-negative bacterium belonging to the family *Pasteurellaceae* and the genus *Haemophilus* (Biberstein, E L; White, 1969). *H. parasuis* is present in pig herds worldwide and it is the causative agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis and meningitis (Amano et al., 1994; Olvera et al., 2006). *H. parasuis* is commonly isolated from nasal cavities, tonsils and the upper part of trachea from both healthy and diseased pigs. Infections with *H. parasuis* are occasional and the clinical disease particularly affects young pigs exposed to a stressful environment (Smart et al., 1989). Clinical signs as coughing, fever, reduced feed intake, lameness, convulsions and septicaemia are features observed with *H. parasuis* infections (Nedbalcova et al., 2006). To date, 15 serovars of *H. parasuis* have been defined, with apparent differences in virulence ranging from highly virulent to non-virulent (Kielstein and Rapp-Gabrielson, 1992). Virulence factors like lipo-oligosaccharides, fimbriae and toxins among others play a critical role in the pathogenesis of *H. parasuis*, however, the precise functions of these factors remains to be fully elucidated (Zhang et al., 2014).

2.2.2.4 *Mycoplasma hyopneumoniae*

Mycoplasma hyopneumoniae is a small bacterium that belongs to the family *Mycoplasmataceae* and the genus *Mycoplasma*. *M. hyopneumoniae* is present in pig herds worldwide and it is the causative agent of enzootic pneumonia, a disease characterized by high morbidity but low mortality. Pigs at all ages are susceptible to enzootic pneumonia and the condition is characterised by a dry and non-productive cough, retarded growth rate and respiratory distress (Dubosson et al., 2004; Thacker, 2004). *M. hyopneumoniae* adhere to the cilia of the respiratory epithelium, causing ciliary damage, clumping and loss of cilia and epithelial cell death. This results in diminished function of the mucociliary apparatus and thereby a weaker airway defence, which can pave the way for secondary infections (DeBey and Ross, 1994).

2.2.2.5 *Mycoplasma hyorhinis*

Mycoplasma hyorhinis is a small bacterium that belongs to the family *Mycoplasmataceae* and the genus *Mycoplasma*. *M. hyorhinis* is present in pig herds worldwide and it adheres to the mucosa of the upper respiratory tract and tonsils. The main clinical disorders associated with *M. hyorhinis* infections are polyserositis and arthritis, while pneumonia and otitis have also been observed. The first clinical signs of infection are sneezing and a clear watery nose fleece, which subsequently will assume a more inflammatory appearance. Normally, the disorder is harmless to the infected animals. Pigs of various ages can be infected but pigs below ten weeks of age are often more prone to infection (Kobisch and Friis, 1996; Thacker and Minion, 2012).

2.2.2.6 *Pasteurella multocida*

Pasteurella multocida is a Gram-negative bacterium belonging to the family *Pasteurellaceae* and the genus *Pasteurella*. *P. multocida* is classified into five serogroups (A, B, D, E and F) and 16 serotypes based on the capsule and lipopolysaccharide antigens, respectively (Carter, 1955; Heddleston et al., 1972). *P. multocida* is found in pigs where it is associated with pneumonia and progressive atrophic rhinitis (PAR). Toxin (PMT) producing strains of *P. multocidia* cause PAR, which is characterized by atrophy of the nasal turbinate bones which, in severe cases, can lead to facial distortion. Toxigenic strains associated with PAR are most frequently of serotype D (Davies et al., 2003; Nielsen et al., 1991). Clinical signs observed with *P. multocida* infection are coughing, fever, sneezing, septicaemia, inflammation of the nasal mucosa and PAR (Davies et al., 2003). *P. multocida* binds to the mucosae of the upper respiratory tract in pigs and several virulence factors like toxins, hemagglutinins, fimbriae, lipopolysaccharides and capsules among others may play a role in the outcome of infection (Okay and Kurt Kizildoğan, 2015).

2.2.2.7 *Streptococcus suis*

Streptococcus suis is a Gram-positive bacterium that belongs to the family *Streptococcaceae* and the genus *Streptococcus*. *S. suis* is found in pig herds worldwide and is associated with arthritis, bronchopneumonia, meningitis and septicaemia in pigs in all ages (Higgins et al., 1990). Clinical signs depend on the pathogenesis of the disease, but symptoms such as anorexia, depression, fever, lassitude, convulsions, incoordination, opisthotonos and tremors have been observed (Staats et al., 1997). To date, 35 serotypes of varying virulence have been described, where serotype 2 is the most prevalent type isolated from diseased pigs (Goyette-Desjardins et al., 2014; Aarestrup et al., 1998).

The natural habitat of *S. suis* is the mucosal surfaces in the upper respiratory tract particular the tonsils and nasal cavities. *S. suis* is a successful colonizer in pigs and pigs can harbour *S. suis* without showing clinical signs, which thereby acts as a reservoir of this pathogen (Staats et al., 1997). Sows carrying the bacterium can infect their litters leading to neonatal death or carrier animals. A number of virulence factors, which are mainly involved in adhesion, anti-phagocytosis, invasion and activation of inflammatory pathways have been described for *S. suis*. However, their role in the pathogenesis of infection has not yet been fully elucidated (Baums and Valentin-weigand, 2009).

2.3 Intestinal pig pathogens

Enteric diseases are a common problem in modern pig production worldwide and can result in severe economic losses due to an elevated use of medication, growth retardation and increased mortality. Enteric diseases can be caused by different pathogens and some pathogens are known to cause disease in young pigs, while others are responsible for disease in older pigs. Stress factors like environmental, nutritional and psychological stress can also have an influence on the susceptibility of the pigs for getting sick by infection (Heo et al., 2013). Selected enteric disease causing pathogens with high impact in the Danish pig industry will be described in the following sections with focus on pathogenesis and clinical signs.

2.3.1 Pathogenesis and clinical signs of virus infections

2.3.1.1 Rotavirus A

Rotavirus A is a non-enveloped, segmented, double-stranded RNA virus, that belongs to the family *Reoviridae* and the genus *Rotavirus* (Paul and Lyoo, 1993). Rotavirus A is endemic in pig herds throughout the world and the virus is associated with acute gastroenteritis, which is usually seen in young animals (Saif and Fernandez, 1996). Rotavirus A infects and replicates in the mature enterocytes located in the mid and upper part of the villi in the small intestine, causing villous atrophy and leading to diarrhoea (Dewey et al., 2003; Lundgren and Svensson, 2001). Diarrhoea can be caused by several mechanisms including malabsorption that occurs secondary to the destruction of enterocytes and stimulation of the enteric nervous system, villus ischemia and secretory components (Lundgren and Svensson, 2003; Ramig, 2004). The incubation time for rotavirus A is usually less than 24 hours, and clinical signs observed are, besides diarrhoea,

dehydration, anorexia and depressed growth, but Rotavirus A can also cause subclinical infections especially in older pigs (Lundgren and Svensson, 2001; Woode et al., 1976).

2.3.2 Pathogenesis and clinical signs of bacterial infections

2.3.2.1 *Brachyspira pilosicoli*

Brachyspira pilosicoli is a Gram-negative bacterium belonging to the family *Brachyspiraceae* and the genus *Brachyspira*. *B. pilosicoli* is found in the intestine and causes porcine intestinal spirochetosis, a milder form of colitis. *B. pilosicoli* binds to the epithelial cells on the colonic mucosa leading to cell shedding and oedema due to functional disruption. Clinical signs of disease include mucus-containing non-bloody diarrhoea, reduced feed conversion and depressed growth rates (Jensen et al., 2000). Infection with *B. pilosicoli* does not seem to be age dependent since it has been isolated from both nursery and finisher pigs and, furthermore, the bacterium can act both as a primary pathogen and as part of mixed infections together with other bacteria (Jacobson et al., 2003; Pedersen et al., 2014; Thomson et al., 1998).

2.3.2.2 *Escherichia coli*

Escherichia coli is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae.* Enterotoxinogenic *E. coli* (ETEC) is one of the *E. coli* strains, which can cause enteric disease. A characteristic of ETEC strains are the fimbrial adhesins, which allow attachment to mucosal surfaces in the intestine. ETEC expressing F4 (also known as K88) and F18 fimbrial adhesins (F4 ETEC and F18 ETEC) can cause diarrhoea in pigs and they are responsible for post-weaning diarrhoea, while F4 ETEC is also associated with diarrhoea in newborn piglets (Frydendahl, 2002; Ojeniyi et al., 1994). F4 ETEC and F18 ETEC adhere with their fimbriae to the intestinal brush border to specific F4 and F18 receptors, respectively, and the susceptibility of pigs to these pathogens is determined by the presence of F4 and F18 receptors (Nguyen et al., 2017). Post-weaning diarrhoea occurs a few weeks after weaning and clinical signs vary from a mild disease with inappetence to watery diarrhoea in severe cases, where suddenly dead also can occur. In general, diarrhoea and a bluish-red discolouration of the skin is observed with post-weaning diarrhoea (van Beers-Schreurs et al., 1992).

2.3.2.3 *Lawsonia intracellularis*

Lawsonia intracellularis is a Gram-negative bacterium that is classified in the delta subdivision of the *Proteobacteria* (Mcorist et al., 1995). *L. intracellularis* is widespread in pig herds worldwide and it is an obligate intracellular bacterium that replicates in the enterocytes. The bacterium can cause proliferative enteropathy, which is a common disease of weaned pigs and it is characterized by thickening of the terminal ileum, caecum and colon mucosa as a result of adenomatous proliferation of immature enterocytes. The disease can be mild and self-limiting but sometimes it can cause persistent diarrhoea, reduction in weight gain, haemorrhagic enteropathy and sudden dead (Lawson and Gebhart, 2000; McOrist et al., 2006; Stege et al., 2004). *L. intracellularis* is primarily associated with diarrhoea in growers and finishers (Jacobson et al., 2003; Stege et al., 2000).

2.4 Diagnostic methods for detection of veterinary pathogens

Traditionally, laboratory diagnostics for detection of veterinary pathogens have relied on methods like culturing, visualizing and antibody and antigen detection. However, in the more recent decades, methods for detection of nucleic acid such as the polymerase chain reaction (PCR) have been developed for diagnostic use. In order for a method to be a reliable, accurate and useful diagnostic tool it should fulfil certain prerequisites including speed, simplicity, sensitivity, specificity, reproducibility and low cost (Deb and Chakraborty, 2012; Schmitt and Henderson, 2005).

2.4.1 Sampling and sample types

The chance of detecting a given pathogen depends largely on the correct collection of samples. A sample must be taken from an appropriate site on the animal and at the right time. The site from which the sample is collected is influenced by the knowledge of the pathogenesis of the suspected pathogen(s). The right time for pathogen detection is usually as soon as possible after the affected animal shows clinical signs, since at this time the pathogen load will be at its maximum. Samples should be sent to the laboratory as fast as possible after the collection, and furthermore, must be kept cold and stored in an appropriate media or buffer in relation to the suspected pathogen(s).

The sample types collected depend on the animal. For living pigs, nasal swab, oral fluid, faecal, blood and air samples are the most typical types of sample. Additionally, tissue samples taken from the part of the body where lesions are observed can be used if the pig is dead. The type of sample also depends on the kind of diagnostics performed. Oral fluid and faecal samples are suitable when

conducting pen based or herd level diagnostics. By using these sample types, it is easy to obtain one sample per. pen, which (theoretical) should contain sample material from all the pigs in a pen. Oral fluid samples can be collected through a cotton rope placed in the affected pens. The pigs are allowed to chew on the rope for approximately 30 minutes, after which the oral fluid can be extracted from the cotton by twisting it in a plastic bag (Biernacka et al., 2016). Faecal samples can be collected from socks covering the boots of a person walking through the contaminated part of the pen, by which faecal matter will be collected on the socks (Figure 5) (Pedersen et al., 2015). Air samples, which are collected by an air sampler, are also useful in herd level diagnostics (Stark et al., 1998). For single animal diagnostics, nasal swab and blood samples are suitable. However, with this kind of sampling the veterinarian needs to be in contact with each of the tested pigs to obtain the samples. Thus, this kind of sampling types can be more time consuming and expensive, depending on the number of sampled animals. Therefore, the choice of sample type should be based on clinical signs, on the knowledge of the pathogenesis of the suspected pathogen(s) and on the number of diseased animals.

Figure 5 Picture showing sampling of faeces (Pedersen et al., 2015).

2.4.2 Classical methods – cultivation, antibody and antigen detection

Some of the oldest diagnostic methods are cultivation-based methods, which are still used in many laboratories today. In recent years, these methods have been improved considerably with advances in the diversity of media components, use of heterologous hosts, use of organ culture and tissue explants, controlling of environmental conditions and use of growth-promoting factors. Consequently, cultivation-based methods have been useful in discovering and identifying new or unanticipated pathogens (Relman, 1999). Cultivation of pathogens are usually simple, however, it can be a time consuming process taking between two to three days before the initial results are

verified through a confirmation process. In addition, the cultivation-based methods depend on the ability of the pathogen to grow in a medium (Zhao et al., 2014).

For antigen-based detection, methods like immunofluorescence and immunohistochemical staining and enzyme-linked immunosorbent assay (ELISA) are used. The big revolution within these methods came with the development of monoclonal antibodies, which are highly specific in their binding to antigen. Immunofluorescence and immunohistochemical staining relies on similar principles, in which the binding of an antigen to an agent-specific antibody is detected by the emission of light. For immunofluorescence staining, a fluorescent-labelled antibody is used, while an enzyme-labelled antibody is used in the immunohistochemical staining. In the ELISA test, the antigen is also recognized by an agent-specific antibody, and binding will result in a visible colour change. Here, the amount of antibody-antigen binding can be measured by a spectrophotometer. Furthermore, the availability of commercially ready-to-use ELISA kits for veterinary practices allows for a large number of samples to be analysed quickly. However, an important notion to make when using antigen detection methods is the fact that they are agent specific and since antigens can be altered due to e.g. mutations this can lead to loss of binding affinity and thereby to loss of test sensitivity because of false-negative results (Clarke and Casals, 1958; Donaldson, 2015; King et al., 1997).

Detection of antibody responses can be a valuable test for defining the infection status of an animal. In this context, the antibody test can be used to determine whether an animal has suffered from any previous infections caused by a particular pathogen, to see if the animal has responded to a vaccination or to see if a specific pathogen is linked to a clinical event. Methods like ELISA, neutralisation and complement fixation can be used for antibody detection and in these methods serum antibody interacts with the agent of interest. In the ELISA test, the antibody-antigen interaction can be followed by the presence of a colour change due to the chemical reaction of an indicator enzyme, while in the neutralisation test the interaction is based on the presence of cytopathic effect on the cell culture. In the complement fixation test, a positive sample containing antibodies will result in non-haemolysis of the red blood cells due to fixation of the complement as a result of antibody-antigen interaction (Schmitt and Henderson, 2005). Furthermore, some viruses, including IAVs, have the ability to haemagglutinate and thereby, the haemagglutination inhibition (HI) test can be used for diagnosis of infection. Based on this test the HI titer, which is the highest dilution of serum that inhibits the agglutination of the red blood cells, can be found (Clarke and Casals, 1958). An antibody response should be considered in relation to the vaccination status of the

animal, thus, sero-responses to vaccines should be distinguished from those that occur after a natural infection.

2.4.3 Polymerase chain reaction (PCR)

In recent years, the methodology used for analysing of veterinary samples has shifted to the use of molecular biology techniques, which allow characterization on the genome level. The molecular technique with the widest application in veterinary diagnostics is polymerase chain reaction (PCR) and the strength of this method is its ability to make millions of copies of a target gene (Schmitt and Henderson, 2005). PCR was developed in the 1980s and today it is one of the most used technologies in the world (Saiki et al., 1988). PCR is a highly sensitive and specific method that allows detection of a pathogen in a sample by detecting a specific stretch of nucleic acids. It operates by amplifying a specific gene sequence in a cyclic process by using a pair of synthetic oligonucleotide primers. The primers, which are usually about 18 to 22 residues long, are termed forward and reverse primer and these are complementary to the target DNA and define the region to be amplified. In the first step of PCR, the double-stranded target DNA is denatured at high temperature resulting in single-stranded DNA. This allows for the forward and reverse primers to anneal to the complementary sequence in the target DNA at a lower temperature. The annealing is followed by an elongation step in which the primers are extended in the presence of deoxynucleotides triphosphates (dNTPs) and a thermostable DNA polymerase, which adds the dNTPs complementary to the target sequence in the 5’ to 3’ direction. The primer extension products synthesized in one cycle will serve as templates in the next cycle and by repeating this thermal cycling between 35 to 45 times, the amount of target DNA will increase exponentially and millions copies of the target DNA will be obtained (Figure 6). PCR can also detect RNA sequences by using the method reverse transcription PCR (RT-PCR). RT-PCR contains a preliminary step where RNA is reverse transcribed into complementary DNA (cDNA) using a reverse transcriptase. The synthesized cDNA is then used as template for amplification in the PCR thermal cycling process. The amplified PCR product can be detected by agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV light. In the agarose gel, the amplicons are separated based on size and this gel-based PCR method is termed conventional PCR (Freeman et al., 1999; Schochetman et al., 1988; Smith, 1996).

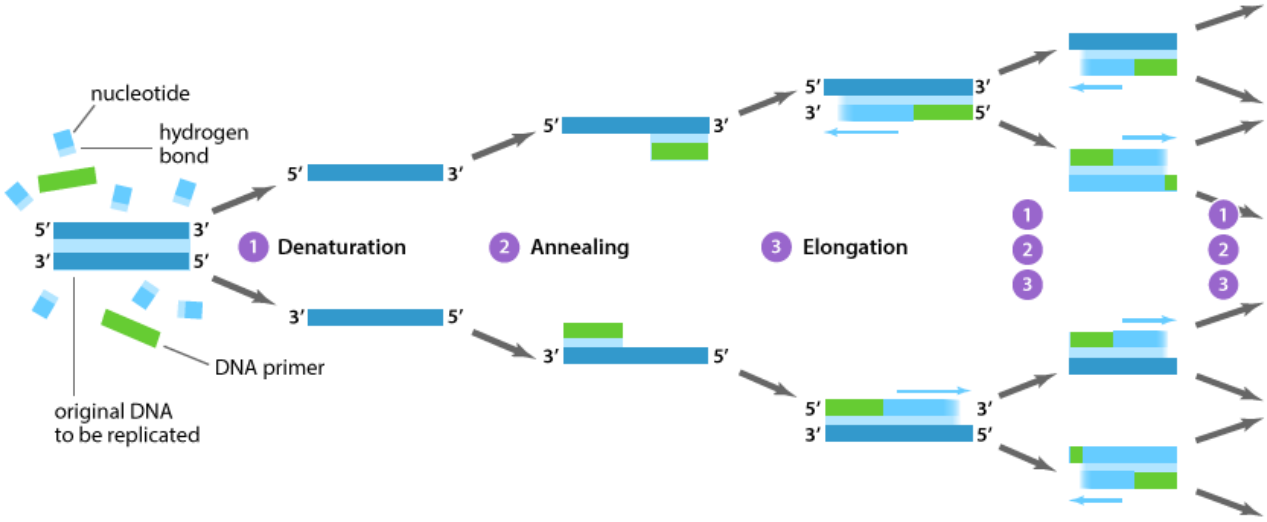

Figure 6 Schematic overview of polymerase chain reaction (PCR). 1) The double-stranded DNA template is denatured. 2) PCR primers anneal to the single-stranded DNA template. 3) The thermostable DNA polymerase elongates the PCR primers by addition of nucleotides resulting in a double-stranded product complementary to the target sequence. Figure obtained from (Nair, 2014).

2.4.3.1 Quantitative real-time PCR

In contrast to conventional PCR, in which the results are obtained at the end of the reaction, quantitative real-time PCR (qPCR) or reverse transcription qPCR (RT-qPCR) displays the data of amplification after each cycle. PCR becomes real-time by the addition of either a fluorescent dye or a fluorogenic probe. Fluorescent dyes, such as SYBR green I and EvaGreen, bind to the double-stranded DNA, thereby emitting a fluorescent signal. There are several types of probe designs available, but the most common type is the dual-labelled probes, also known as hydrolysis probes. These probes, which anneals between the PCR primers, have a fluorescent reporter dye at the 5' end and a quencher at the 3' end and are typically between 20 to 30 nucleotides long. During elongation, the dual-labelled probe is hydrolysed by the 5´-3´exonuclease activity of the DNA polymerase, which cleaves the fluorescent reporter from the probe resulting in generation of a fluorescent signal (Figure 7). The fluorescent signal, emitted from both dye labelling systems, is detected at the end of each PCR cycle and as the number of amplicons increase during the qPCR reaction, the fluorescent signal also increases. The progression of the qPCR reaction is visualized by plotting the fluorescent signal against the cycle number giving rise to the amplification curve. Based on a defined threshold the quantification cycle (C_q) value, which is the cycle number at which the amplification curve for a given sample crosses the threshold, can be measured. A low C_q value indicates a high template concentration, while a high C_q value indicates a low template concentration. In qPCR, the targeted

nucleic acid concentration can be quantified either relative to a reference or to a set of standards used to construct a standard curve. A standard curve consisting of a series of samples with known amounts of target template can also be used to determine the performance of a qPCR assay in that it allows for estimation of the efficiency, detection of the assay dynamic range and limit of detection. By plotting the C_q values of the samples from the standard curve against the $\log_{10}$ of the sample concentrations, a linear curve with a negative slope is expected. Based on this plot, the efficiency can be calculated by the equation: efficiency = $10^{(-1/slope)}$-1. In theory, the amount of qPCR product is doubled after each cycle, which should yield an efficiency of 100%. However, both higher and lower efficiencies can be obtained, which can be caused by e.g. inhibitors of the qPCR or the lack of optimal prime/probe binding (Bustin et al., 2009; Svec et al., 2015).

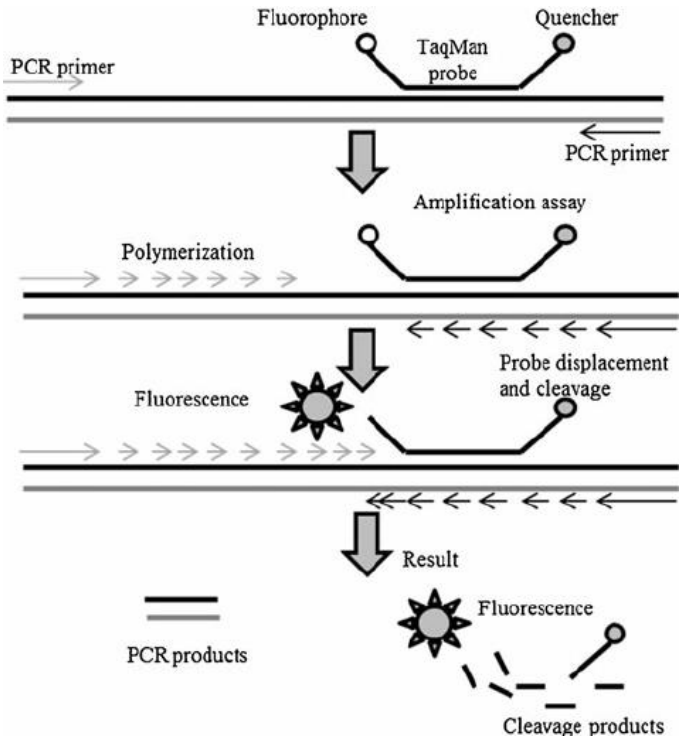

Figure 7 Schematic overview of quantitative real-time polymerase chain reaction (qPCR). Figure obtained from (Botes et al., 2013).

Probe-based qPCR is the most common technology used in diagnostic analyses due to its higher specificity compared to dye-based qPCR, which can bind to any double stranded product, including primer-dimers, and with that can give rise to false-positive results. This problem can to some extent be remedied by a melting curve analysis, which can differentiate the target products from the primer-dimer formation. Probe-based qPCR is more expensive than dye-based qPCR due to the high cost of the probe. However, since the probe-based assay allows sequence specific binding and

thereby eliminates false-positive results this type of assay is preferred in many contexts (Law et al., 2015; Peters et al., 2004).

Multiplexed qPCR or RT-qPCR tests that allows for simultaneous screening of several pathogens from a single sample is one of the newest tools developed within the recent years. In multiplex qPCR, several sets of specific primers and probes are used, while only one set of specific primers and probe are used in a "single" qPCR assay. Multiplex qPCR can be very useful in evaluation of samples from disease complexes like in cases with respiratory or reproductive problems in a pig herd (Xu et al., 2012). However, in order to produce a successful multiplex qPCR, it is important to be aware that the included assays have to be carried out under the same PCR conditions, which imposes certain design requirements of the primers and probes.

2.4.3.2 High-throughput real-time PCR

qPCR can also be performed in a high-throughput manner by using platforms in which a high number of different samples can be tested in a high number of different assays. The high-throughput qPCR BioMark™ system from Fluidigm (South San Francisco, USA) is one of the available platforms on the market and represents a system capable of performing parallel qPCR reactions in a microfluidic manner. The reactions are carried out in Dynamic Array™ (DA) Integrated Fluidic Circuit (IFC) nanofluidic chips, which contain fluidic networks that automatically combine the samples with the assays (Figure 8). The BioMark dynamic array system allows its users to combine either 48 samples with 48 assays (48.48DA), 96 samples with 96 assays (96.96DA) or 192 samples with 24 assays (192.24DA) resulting in 2,304, 9,216 or 4,608 individual reactions, respectively, in a single run. The samples and qPCR reagents are loaded into appertaining inlets in the DA chip and by pressure these are distributed into the reaction chambers via microfluidic channels prior to the qPCR (Spurgeon et al., 2008). The high-throughput qPCR BioMark system has been widely used in research studies for years, including e.g. the study of innate immune response to IAV (Skovgaard et al., 2013). More recently, the system has also been used as a screening and detection tool for food- and waterborne pathogens and for tick-borne diseases (Ishii et al., 2013; Michelet et al., 2014).

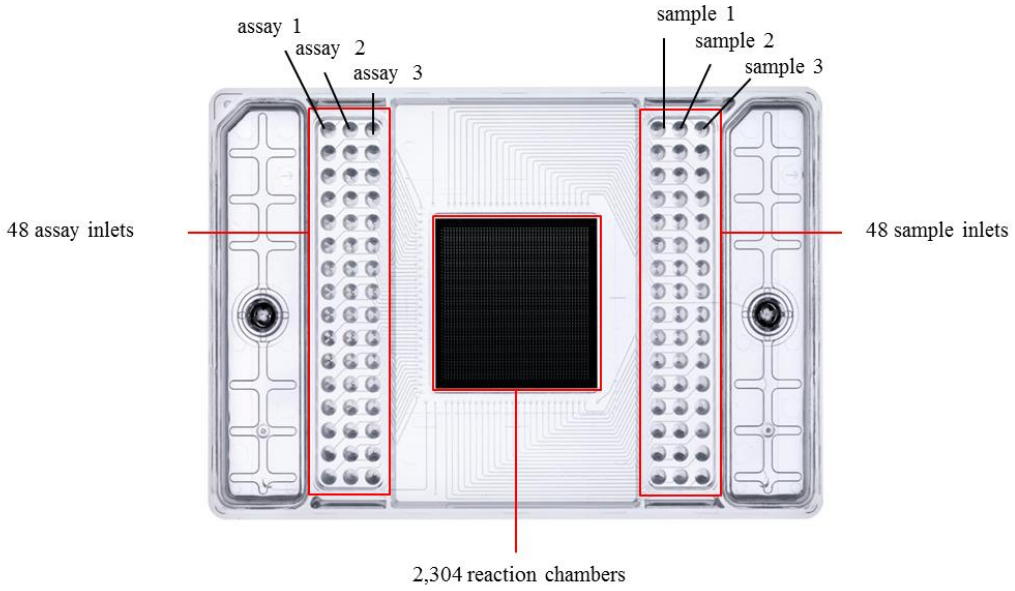

Figure 8 48.48 Dynamic array IFC chip. Picture modified from the Fluidigm website.

2.4.4 Nucleic acid sequencing

Nucleic acid sequencing technologies have brought insights and opportunities into understanding all types of biological phenomena including the diversity of veterinary pathogens. Sequencing is a process of determining the precise order of nucleotides within a DNA or RNA (cDNA) molecule. One of the first sequencing methods was the Sanger sequencing method, also known as dideoxy chain termination method, which was developed by Frederick Sanger in 1977 (Sanger et al., 1977). The principle behind the modem Sanger sequencing method relies on the use of fluorescent labelled nucleotides that have the ability to terminate primer-dependent elongation followed by high resolution gel separation of the fragments. The sequence of the template is determined based on the fragmentation and the emissions produced by the fluorescent dye labels, which are converted into nucleotide sequence information (Heather and Chain, 2016). Sanger sequencing is commonly used today due to its simplicity and ease of use, and this method is preferable when sequencing amplicon targets up to 1-500 base pairs and sequencing single genes due to the use of specific primers (Deurenberg et al., 2017). Over the last decades, next-generation sequencing (NGS) has evolved rapidly and with this method millions of fragments can be sequenced in a single run in contrast to Sanger sequencing which only produces one forward and reverse read. Today, a number of different NGS platforms using different sequencing technologies are available and properties like cost,

output and fragment length are important factors for these. NGS allows sequencing of the whole genome of numerous pathogens in a single run without using target specific primers and it can sequence more than 100 genes simultaneously (Behjati and Tarpey, 2013).

Sequence analyses can be used to answer diagnostic questions such as the genetic relationship within pathogens and the detection of mutations in genomes, which can be important knowledge since mutations within a gene can lead to resistance against antibiotics or antivirals (Deurenberg et al., 2017). Alignments and phylogenetic analyses based on sequences of a specific pathogen or gene can contribute with indispensable information about the evolutionary relationship based on similarities and differences of the sequences. In addition, sequencing of the 16S rRNA gene is commonly used to study the phylogeny and taxonomy of bacteria (Janda and Abbott, 2007).

2.4.5 Point of care diagnostics

Point of care (POC) diagnostics has attracted increased attention in the recent years. POC testing has the advantage that immediate results can be obtained since the test is carried out on site or near to the site of the animal (St-louis, 2000). Thereby, it is not necessary to send the samples to the laboratory for analysis and await the results, which can take hours or days. The use of POC testing for diagnostics is also interesting due to the potential of decreasing cost and reduction in the time between sampling and results, allowing for a faster treatment decision. Several POC devices have been developed and different technologies have been used. Some of the currently available veterinary POC tests offer cost-effective and decentralised diagnosis of a wide range of infectious diseases, however, these tests come with some limitations. In general, the analytical performances of several POC tests are still considered limited compared to laboratory-based testing, but the use of microfluidic technology has brought new opportunities into the world of POC diagnostics (Busin et al., 2016).

2.4.6 Interpretation of laboratory findings

It is crucial that results obtained by analytical methods are accurate and reliable, since diagnoses and treatments are based on results obtained by these methods. False-positive and false-negative results can occur in many tests and therefore, the inclusion of positive and negative controls is important in every single test. Rapid detection methods, which are highly sensitive and specific are important in the veterinary industry to prevent outbreaks of diseases and the spread of pathogens. Furthermore, sensitivity is important since a single copy of a pathogen present has the risk to cause

infection. Methods get more and more sensitive resulting in the ability to detect latent and low level persistent infections, which do not always necessarily result in disease. Therefore, interpretation of the results obtained by these very sensitive methods should be taken with care. Additionally, high specificity is important to be able to differentiate between different pathogen species and between very similar pathogens. For detection of a specific pathogen, the use of one method can be sufficient, while combination of several methods is necessary in other cases depending on the type of pathogen and on the purpose of the detection. Each method has its own advantages and limitations, which should be seen in relation to the use of the specific method.

3 Own studies

3.1 Manuscript I

Subtyping of swine influenza viruses using a high-throughput real-time PCR platform

Nicole B. Goecke[1*], Jesper S. Krog[1], Charlotte K. Hjulsager[1], Kerstin Skovgaard[1], Timm C. Harder[2], Solvej Ø. Breum[1], Lars E. Larsen[1]

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Subtyping of Swine Influenza Viruses Using a High-Throughput Real-Time PCR Platform

Nicole B. Goecke[1], Jesper S. Krog[1], Charlotte K. Hjulsager[1], Kerstin Skovgaard[1], Timm C. Harder[2], Solvej Ø. Breum[1] and Lars E. Larsen[1]*

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Influenza A viruses (IAVs) are important human and animal pathogens with high impact on human and animal health. In Denmark, a passive surveillance program for IAV in pigs has been performed since 2011, where screening tests and subsequent subtyping are performed by reverse transcription quantitative real-time PCR (RT-qPCR). A disadvantage of the current subtyping system is that several assays are needed to cover the wide range of circulating subtypes, which makes the system expensive and time-consuming. Therefore, the aim of the present study was to develop a high-throughput method, which could improve surveillance of swine influenza viruses (swIAVs) and lower the costs of virus subtyping. Twelve qPCR assays specific for various hemagglutinin and neuraminidase gene lineages relevant for swIAV and six assays specific for the internal genes of IAV were developed and optimized for the high-throughput qPCR platform BioMark (Fluidigm). The qPCR assays were validated and optimized to run under the same reaction conditions using a 48.48 dynamic array (48.48DA). The sensitivity and specificity was assessed by testing virus isolates and field samples with known subtypes. The results revealed a performance of the swIAV 48.48DA similar to conventional real-time analysis, and furthermore, the specificity of swIAV 48.48DA was very high and without cross reactions between the assays. This high-throughput system provides a cost-effective alternative for subtyping of swIAVs.

Keywords: swine influenza virus, subtyping, surveillance, real-time PCR, high-throughput real-time PCR, diagnostics

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INTRODUCTION

Swine influenza is a respiratory disease caused by multiple subtypes of influenza A virus (IAV). The genome of IAV consists of eight segments, which code for different virus proteins. Subtype classification of IAV is based on the encoded surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), and so far, 16 different HA and nine different NA subtypes have been described together with two recently discovered bat-derived subtypes, H17N10 and H18N11 (Cheung and Poon, 2007; Wu et al., 2014). Influenza A virus contains further six "internal" gene segments which encode basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), nucleoprotein (NP), matrix (M1, M2), and non-structural proteins (NS1, NS2). These segments and their translation products have an essential role in the virulence and host specificity of a given IAV and can also impact the risk of transmission to humans (Bi et al., 2015).

The predominant swine IAV (swIAV) subtypes globally are H1N1, H3N2, and H1N2, which all show considerable diversity. The genetic and antigenic characteristics of IAVs in pigs differ depending on their geographic locations (Kuntz-Simon and Madec, 2009; Simon et al., 2014). In Europe, the dominant H1N1 swIAV is of avian origin, referred to as avian-like swine H1N1 ($H1_{av}N1_{av}$), which was introduced from waterfowl to pigs in the late 1970s (Pensaert et al., 1981; Simon et al., 2014). The dominant genotype of H3N2 virus in European pigs is the H3N2 ($H3_{sw}N2_{sw}$) virus that was introduced in 1984. The HA and NA genes of the $H3_{sw}N2_{sw}$ are of human origin, while the other six gene segments are of avian ($H1_{av}N1_{av}$) descent (Castrucci et al., 1993). In 1994, an H1N2 reassortant was isolated for the first time in United Kingdom and has subsequently been detected in many European countries. This human-like reassortant swine H1N2 ($H1_{hu}N2_{sw}$) virus comprised the HA gene from a human seasonal H1N1 virus, the NA gene from the $H3_{sw}N2_{sw}$ virus and internal genes from the $H1_{av}N1_{av}$ virus (Alexander et al., 1998). The dominating European $H1_{hu}N2_{sw}$ virus has never been detected in Denmark, however, a new reassortant $H1_{av}N2_{sw}$, containing the HA gene from the $H1_{av}N1_{av}$ virus and the NA gene from $H3_{sw}N2_{sw}$, was found in Denmark in 2003 (Trebbien et al., 2013). This avian-like H1N2 ($H1_{av}N2_{sw}$) virus has become established in Denmark and other European countries (Trebbien et al., 2013; Simon et al., 2014) and is now the most prevalent subtype circulating in Danish pigs. In 2009, a new human pandemic strain [A(H1N1)pdm09] entered the global swine population and is now enzootic in swine globally. Furthermore, an increasing number of reassortants between the predominant enzootic swIAVs and the A(H1N1)pdm09 virus have been observed, making subtyping of swIAV a very complex task (Starick et al., 2011; Watson et al., 2015). Furthermore, spillover of seasonal human H3 ($H3_{hu}$) segments and human N2 ($N2_{hu}$) have been observed in Danish swine (Breum et al., 2013; Krog et al., 2017).

In Denmark, a passive surveillance program for swIAVs has been conducted since 2011. A requirement for efficient swIAV surveillance is highly sensitive and specific diagnostic tests. Today, the swIAV screening test and subsequent subtyping is performed by reverse transcription (RT) quantitative real-time PCR (qPCR), where several different assays are needed to cover the wide range of circulating subtypes, which make detection and subtyping costly and time consuming. The aim of the present study was to establish a high-throughput method for detection and subtyping of swIAVs in Danish pigs. The BioMark dynamic array (DA) (Fluidigm, South San Francisco, USA) is capable of performing parallel qPCRs by combining e.g., 48 samples with 48 assays or 96 samples with 96 assays in a combinatorial manner inside the integrated fluidic circuit (IFC) resulting in either 2,304 or 9,216 individual reactions in a single run. Besides being able to process a high number of reactions in a single run, the high-throughput qPCR BioMark system also uses less sample and reagent volume compared to standard qPCR platforms (Spurgeon et al., 2008). The present study describes the design, optimization and validation of a swIAV 48.48DA; a setup consisting of 18 qPCR assays targeting the different swIAVs circulating in Europe.

MATERIALS AND METHODS

Samples

In the routine veterinary diagnostic laboratory at the National Veterinary Institute in Denmark, oral fluid, lung tissue, and nasal swabs are tested for swIAV from pigs with a history of respiratory disease. The samples are tested by an in-house modified RT-qPCR assay detecting the M gene (Trebbien et al., 2013). For selected swIAV positive samples, virus is isolated in Madin-Darby Canine Kidney (MDCK) cell cultures, followed by full genome sequencing by Next Generation Sequencing (NGS) (Krog et al., 2017). For validation of the swIAV 48.48DA a total of 32 field samples from 2015 and 2016 (**Table 1**) and 29 virus isolates for which full genome sequences were available were used (**Table 2**).

Primer and Probe Design

The swIAV 48.48DA was designed to include qPCR assays targeting the different lineages of H1, H3, N1, and N2 circulating in pigs in Europe. For the H1 subtypes the design aimed at differentiating between the H1 lineages; $H1_{av}$, H1 from A(H1N1)pdm09 ($H1_{pdm}$) and $H1_{hu}$. For the H3 lineages the aim was to differentiate between $H3_{sw}$ and $H3_{hu}$. For the NA subtypes N1 and N2 broadly reacting assays ($N1_{B1}$, $N1_{B2}$, $N2_{B1}$, $N2_{B2}$) were included together with an assay specifically detecting the A(H1N1)pdm09 lineage of N1 ($N1_{pdm}$) and an assay specifically detecting $N2_{hu}$ derived from the seasonal human H3N2, that circulated in humans in the mid-1990s. Accordingly, $N1_{pdm}$ positive viruses gave positive results with the $N1_{B1}$, $N1_{B2}$ and $N1_{pdm}$ assays, while $N2_{hu}$ positive viruses gave positive results with the $N2_{B1}$, $N2_{B2}$ and $N2_{hu}$ assays.

In addition, six qPCR assays specific for the internal genes of A(H1N1)pdm09 ($PB1_{pdm}$, $PB2_{pdm}$, PA_{pdm}, NP_{pdm}, M_{pdm}, NS_{pdm}) were included. Primers and probes were either selected from previously published methods or designed in the present study. The final sets of primers and probes consisting of 18 PCR assays, of which 12 were designed *de novo*, two were from published literature, three were modified published assays and one was an in-house assay. The modifications are highlighted in bold in **Tables 3**, **4**. New primer and probe sequences were designed based on alignments comprising full-length sequences of the eight gene segments from European swIAVs. The sequences were retrieved from Influenza Research Database[1] The specificity of primers and probes was tested *in silico* by using BLAST search (Altschul et al., 1990), while melting temperature of the oligonucleotides was approximated using the online tool "OligoCalc" (Kibbe, 2007). The RT-qPCR assays were tested on the Rotor-Gene Q qPCR system (QIAGEN, Hilden, Germany) using a panel of six strains of cultured viruses, representing targets for one or more of the different primer and probe sets. RT-qPCR assays were performed in a final volume of 25 μL using QIAGEN OneStep RT-PCR kit (QIAGEN), with 5 μL of 5X QIAGEN One step RT-PCR buffer, 1 μL of 10 mM nucleotides dNTP mix, 1.25 μL of 25 mM $MgCl_2$, 1 μL of 100 μM primers, 0.25 μL of 30 μM probe, 1 μL QIAGEN enzyme mix, 2 μL RNA and 12.5 μL RNase-free water. Thermal cycling conditions

[1]http://www.fludb.org

TABLE 1 | HA and NA subtyping of swIAV from Danish field samples by swIAV 48.48DA.

Sample name	Origin[a]	Subtype[b]		Singleplex[c]		swIAV 48.48DA[d]									
		HA	NA	M	M	$H1_{av}$	$H1_{hu}$	$H1_{pdm}$	H3	$N1_{B1}$	$N1_{B2}$	$N1_{pdm}$	$N2_{B1}$	$N2_{B2}$	$N2_{hu}$
A/Swine/Denmark/7961-7/2016	?	H1pdm	N1pdm	21	22.41			27.18		27.87	21.09	**24.52**	**30.66**		
A/Swine/Denmark/9186-1/2016	ns	H1pdm	N1pdm	29.57	28.73			22.53		25.84	29.97	29.45			
A/Swine/Denmark/10130-1/2016	ns	H1pdm	N1pdm	22	22.53			19.95		21.58	22.25	21.43			
A/Swine/Denmark/16219-1/2016	?	H1pdm	N1pdm	23	25.90			23.57		25.93	26.49	26.92			
A/Swine/Denmark/16966-2/2016	?	H1pdm	N1pdm	25.85	26.34			24.11		24.96	25.46	26.99			
A/Swine/Denmark/19295-1/2015	lu	H1pdm	N1pdm	21.50	21.43			15.93		18.76	19.80	19.53			
A/Swine/Denmark/19089-3/2015	lu	H1pdm	N1pdm	21	22.24			18.97		19.27	20.27	21.66			
A/Swine/Denmark/19090-1/2015	lu	H1pdm	N1pdm	24	25.58			20.05		25.63		23.61			
A/Swine/Denmark/20835-1/2015	?	H1pdm	N1pdm	24	23.78			23.83		21.52	22.32	22.18			
A/Swine/Denmark/6521-1/2016	ns	H1pdm	N2sw	27	24.99			26.42					28.24	32.63	
A/Swine/Denmark/7988-2/2016	lu	H1pdm	N2sw	18	18.50	**27.74**		**22.10**					24.92	25.79	
A/Swine/Denmark/9154-4/2016	ns	H1pdm	N2sw	26.44	26.18			23.28					27.83	32.56	
A/Swine/Denmark/20566-1/2015	ns	H1pdm	N2sw	19	19.10			14.89					26.71	31.26	
A/Swine/Denmark/10856-3/2016	ns	H1av	N1	26	25.72	31.59				29.11					
A/Swine/Denmark/23293-4/2015	ns	H1av	N1	25	24.47	32.85				25.02	26.57				
A/Swine/Denmark/8938-1/2015	ns	H1av	N?→N1	31	25.53	23.91					24.95				
A/Swine/Denmark/6392-2/2016	ns	H1av	N2sw	21.39	23.45	22.64							26.43		
A/Swine/Denmark/6469-1/2016	lu	H1av	N2sw	20	21.94	28.10							27.24		
A/Swine/Denmark/6534-3/2016	lu	H1av	N2sw	15.87	18.78	18.18							22.06		
A/Swine/Denmark/6598-1/2016	sa	H1av	N2sw	29	26.30	28.91									
A/Swine/Denmark/6637-1/2016	ns	H1av	N2sw	25	25.68	25.54							25.26		
A/Swine/Denmark/6686-1/2015	ns	H?→H1av	N2sw	30	25.25	24.09								34	
A/Swine/Denmark/8065-1/2016	ns	H1av	N2sw	22	23.62	24.37								28.77	
A/Swine/Denmark/9051-1/2016	lu	H1av	N2sw	18.75	20.92	19.80							25.17	29.47	
A/Swine/Denmark/9846-1/2016	ns	H1av	N2sw	25.84	25.35	25.24							29.89		
A/Swine/Denmark/11013-3/2016	lu	H1av	N2sw	14	14.34	16.52							17.67	20.67	
A/Swine/Denmark/14170-2/2016	?	H1av	N?→N2sw	25.41	23.51	23.95							27.94	31.68	
A/Swine/Denmark/15963-2/2016	?	H1av	N2sw	23	22.49	20.97							30.70	28.21	
A/Swine/Denmark/19292-1/2015	ns	H1av	N2sw	26.45	25.67	23.78							30.37	31.93	
A/Swine/Denmark/19293-1/2015	lu	H1av	N2sw	16.27	17.24	15.38							21.31	25.04	
A/Swine/Denmark/23653-3/2015	ns	H1av	N2sw	27.49	25.35	24.25							30.94	33.87	
A/Swine/Denmark/9079-2/2016	lu	H?	N2sw	27.31	26.39								37.51		

[a] *ns, nasal swab; lu, lung tissue; sa, oral fluid; ?, unknown.*

[b] *Subtype achieved by an in house multiplex RT-qPCR (modified from Henritzi et al., 2016).*

[c] *Cq-value achieved by an in-house modified RT-qPCR assay detecting the M gene (Trebbien et al., 2013).*

[d] *Cq-value achieved by swIAV 48.48DA.*

Samples where a discrepancy was observed between the two analyses are in bold letter.

TABLE 2 | Parallel subtyping of virus isolates by full genome sequencing (left) and qPCR using the swIAV 48.48DA (right).

	Full genome sequencing results									swIAV 48.48DA results							
	H1	H3	N1	N2	M	NP	NS	PA	PB1	PB2	H1	H3	N1	N2	M	NP	NS	PA	PB1	PB2
H1N1																				
A/Swine/Denmark/13-4/2013																				
A/Swine/Denmark/1092-2/2013																				
A/Swine/Denmark/1326-1/2013																				
A/Swine/Denmark/4790-1/2015																				
A/Swine/Denmark/09973-1/2015																				
H1N2																				
A/Swine/Denmark/201-2/2013																				
A/Swine/Denmark/1837-1/2013																				
A/Swine/Denmark/628-3/2014																				
A/Swine/Denmark/02408-1/2015																				
A/Swine/Denmark/03572-1/2015																				
A/Swine/Denmark/04775-1/2015																				
A/Swine/Denmark/05758-1/2015																				
A/Swine/Denmark/08913-3/2015																				
H1N1pdm																				
A/Swine/Denmark/365-3/2014																				
A/Swine/Denmark/9477-1/2014																				
A/Swine/Denmark/03655-3/2015																				
A/Swine/Denmark/05736-1/2015																				
A/Swine/Denmark/05775-2/2015																				
H1N2hu																				
A/Swine/Denmark/616-3/2014																				
A/Swine/Denmark/4811-10/2015																				
H1pdmN2sw																				
A/Swine/Denmark/176-1/2012																				
A/Swine/Denmark/6252-2/2014																				
A/Swine/Denmark/10781-1/2014																				
A/Swine/Denmark/03627-2/2015																				
A/Swine/Denmark/04804-3/2015																				
A/Swine/Denmark/20566-1/2015																				
H1pdmN2hu																				
A/Swine/Denmark/00798-2/2015																				
A/Swine/Denmark/10377-1/2015																				
H3hu05N2sw																				
A/Swine/Denmark/15164-1/2014																				
Color code																				
v	Swine influenza (H1N1, H1N2, H3N2) origin				H1N1pdm origin						Humane H3N2					Not sequenced				

Samples where a discrepancy was observed between the two analyses are in bold letter.

TABLE 3 | Primers and probes for detection of M, HA, and NA genes.

Primer/probe	Sequence (5′-3′)	Product size (bp)	References
$H1_{av}$			Modified from (Henritzi et al., 2016)[a]
$H1_{av}$-F	GAAGGRGGATGGACAGG**A**AT**G**A	139	
$H1_{av}$-R	CAATTAHTGARTTCACTTTGTTG**CTG**		
$H1_{av}$-P	**FAM**-TCTGGTTACGCAGCWGATCAGAAAA-BHQ1		
$H1_{hu}$		169	Primer: This study
$H1_{hu}$-F	GGWTGGTATGGTTATCATCAT		Probe: (Bonin et al., 2018)
$H1_{hu}$-R	CTCGATTACAGAGTTCACC		
$H1_{hu}$-P	FAM-CAGGGATCTGGCTATGCTGCAGAYC-BHQ1		
$H1_{pdm}$		87	In-house assay
$H1_{pdm}$-F	AGTTCAAGCCGGAAATAGCA		
$H1_{pdm}$-R	CCCGGCTCTACTAGTGTCCA		
$H1_{pdm}$-P	FAM-CCCAAAGTGAGGRATCAAGAAGGGAG-BHQ1		
$H3_{hu}$		93	This study
$H3_{hu}$-F	TGATGGAGAAAACTGCACACTA		
$H3_{hu}$-R	CGTTCAACAAAAAGGTCCCATTTC		
$H3_{hu}$-P	FAM-CACACTGAGGGTCTCCCAATAGAGCATCTA-BHQ1		
$H3_{sw}$		93	This study
$H3_{sw}$-F	TGATGGAGCAAATTGCACACTG		
$H3_{sw}$-R	CGTTCAATGAAAAGGTCCCATTTC		
$H3_{sw}$-P	FAM-CACAATGAGGGTCCCCTAATAGAGCGTCCA-BHQ1		
$N1_{B1}$		99	This study
$N1_{B1}$-F	CCTTGCTTCTGGGTTGAACTAATC		
$N1_{B1}$-R	AGTGTCACTATTTACACCACAAAAGG		
$N1_{B1}$-P	FAM-TGCTCCCGCTAGTCCAGATTGTGTTCTCTT-BHQ1		
$N1_{B2}$		126	Henritzi et al., 2016
$N1_{B2}$-F	AGRCCTTGYTTCTGGGTTGA		
$N1_{B2}$-R	ACCGTCTGGCCAAGACCA		
$N1_{B2}$-P	FAM-ATYTGGACYAGTGGGAGCAGCAT-BHQ1		
$N1_{pdm}$		102	This study
$N1_{pdm}$-F	CGAAATGAGTGCCCCTAATTATC		
$N1_{pdm}$-R	CGATTCGAGCCATGCCAGTTA		
$N1_{pdm}$-P*	FAM-[+C][+C]T[+G]ATTCT[+A]GTGAAATCA[+C]-BHQ1		
$N2_{B1}$		101	This study
$N2_{B1}$-F	TATTGATGAATGAGTTGGGTGTTCC		
$N2_{B1}$-R	ATGCAGCCATGCTTTTCCATC		
$N2_{B1}$-P	FAM-TGAACTGGACCATGCTATACACACTTGCCT-BHQ1		
$N2_{B2}$		116	Modified from (Henritzi et al., 2016)[a]
$N2_{B2}$-F	AGTCTGGTGGACYTCAAAYAG		
$N2_{B2}$-R	TTGCGAAAGCTTATATAGVCATGA		
$N2_{B2}$-P	**FAM**-CCATCAGGCCATGAGCCTGWWCCATA-BHQ1		
$N2_{hu}$		92	This study
$N2_{hu}$-F	CTGGTATTTTCTCTGTTGAAGGC		
$N2_{hu}$-R	CCASACTTCAKTTTCCTGYTTCC		
$N2_{hu}$-P*	VIC-T[+C]A[+A]CTCYACATAAAAGCACC[+G]-BHQ1		
M		204	(Loeffen et al., 2011)
M-F	CTTCTAACCGAGGTCGAAACGTA		
M-R	CACTGGGCACGGTGAGC		
M-P	FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1		

[a]*Letters in bold in the sequences indicate the modification compared to the published sequences.*
[*]*Locked Nucleic Acid positions are indicated in brackets.*

TABLE 4 | Primers and probes for detection of the internal pandemic genes of swIAVs.

Primer/probe	Sequence (5′-3′)	Product size (bp)	References
$PB2_{pdm}$			
$PB2_{pdm}$-F	GATAGTAAGCGGGAGAGAC	128	This study
$PB2_{pdm}$-R	GCTGGTTTGCCCTATTGAC		
$PB2_{pdm}$-P	FAM-GCTGAGGCAATAATTGTGGCCATGG-BHQ1		
$PB1_{pdm}$			
$PB1_{pdm}$-F	CAAAGACTACAGATACACATATAG	124	This study
$PB1_{pdm}$-R	ATCTGATACTAATAGCCCTAC		
$PB1_{pdm}$-P	FAM-GGGGAGACACACAAATTCAGACGAG-BHQ1		
PA_{pdm}			
PA_{pdm}-F	GGTGAAAATATGGCACCAGAA	110	This study
PA_{pdm}-R	TGCTAGAGATCTGGGCTC		
PA_{pdm}-P	FAM-GTAGACTTTGATGAYTGCAAAGATGTTGG-BHQ1		
NP_{pdm}			
NP_{pdm}-F	ACGGTCAGCACTCATTCTG	117	This study
NP_{pdm}-R	ACCAGTGAGTACCCTTCC		
NP_{pdm}-P	FAM-TCATGCCCACTTGCTACTGCAAGC-BHQ1		
M_{pdm}			
M_{pdm}-F	CTGGCTAGCACTACRGCA	99	This study
M_{pdm}-R	TACCATYTGCCTAGTCTGATTA		
M_{pdm}-P	FAM-CTCYATGGCCTCTGCTGCCTGT-BHQ1		
NS_{pdm}			
NS_{pdm}-F	GAGGAAATGTCACGAGACTG	119	This study
NS_{pdm}-R	ACTGAAGTTCGCTTTCAGTAC		
NS_{pdm}-P	FAM-TTCCATGACCGCCTGGTCCAATCG-BHQ1		

were as follow: 50°C for 30 min, 95°C for 15 min followed by 40 cycles at 94°C for 10 s, 54°C for 30 s and 72°C for 10 s. The fluorescence signal was acquired at the 54°C step in the Green channel (470–510 nm). Data was analyzed with the Rotor-Gene Q Series Software 2.3.1. (QIAGEN) with the following parameter adjustments: dynamic tube normalization, on; noise slope correction, on; ignore first cycle; outlier removal, 10%; quantification cycle (Cq) threshold fixed, 0.01. All reactions were run in duplicates and non-template control (nuclease-free water) was included in each run.

Primers and the dual labeled probes were purchased from Eurofins Genomics (Ebersberg, Germany), while Locked Nucleic Acid (LNA) probes were from BioNordika (Herlev, Denmark). Primers and probes were stored at −20°C.

RNA Extraction

Viral RNA was extracted from cultured viruses, oral fluid, lung tissue and nasal swab samples by RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Cell culture supernatant, oral fluid and nasal swab samples were prepared by mixing 200 µL material with 400 µL RLT buffer containing β-mercaptoethanol (Sigma-Aldrich, Brøndby, Denmark). Lung tissue samples were prepared by homogenization of 70 mg lung tissue in 1,400 µL RLT buffer containing β-mercaptoethanol (Sigma-Aldrich) on a TissueLyser II (QIAGEN) at 30 Hz in 3 min. The homogenate was centrifuged for 3 min at 12,000 g, and RNA was extracted from of 600 µL of the supernatant. Viral RNA was eluted in 60 µL RNase-free water and stored at −80°C.

cDNA Synthesis and Pre-amplification

cDNA synthesis and pre-amplification of the extracted samples was performed in one step. Briefly, reaction volumes of 25 µL containing 1.50 µL of 10 µM random hexamer (Invitrogen, Carlsbad, California, USA), 0.75 µL primer mix (containing all qPCR primers (200 nM each) listed in **Tables 3**, **4**), 5 µL of 5X QIAGEN One step RT-PCR buffer (QIAGEN), 1 µL of 10 mM nucleotides dNTP mix, 1.25 µL of 25 mM $MgCl_2$, 1 µL QIAGEN enzyme mix, 3 µL sample and RNase-free water were prepared. cDNA synthesis and pre-amplification were performed on a T3 Thermocycler (Biometra, Fredensborg, Denmark) at 50°C for 30 min followed by enzyme inactivation at 95°C for 15 min followed by 24 cycles of 94°C for 10 s, 54°C for 30 s, and 72°C for 10 s. The pre-amplified cDNA was stored at −20°C.

Preparation of the 48.48DA and qPCR

Pre-sample mix was prepared using the following components per sample; 3 µL TaqMan Gene Expression Master Mix (Applied Biosystems, Foster city, USA) and 0.3 µL 20x Sample loading reagent (Fluidigm, South San Francisco, USA). Pre-sample mix (3.3 µL) was mixed with 2.7 µL pre-amplified cDNA. Two different mixes of primers and probes with different concentrations, was prepared for each assay by mixing 3 µL primer/probe-stock (containing either 30 µM of each primer

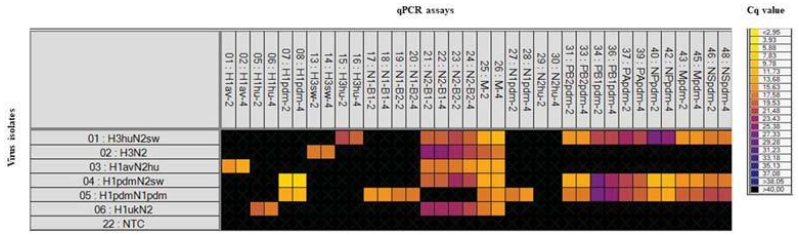

FIGURE 1 | Heat map showing the specificity of the qPCR assays included on the swIAV 48.48DA by testing six virus isolates with known subtype (based on full genome sequencing). Top: The qPCR assays (**Tables 3**, **4**) in two different primer/probe concentrations (indicated by the numbers two or four). Left: The virus isolates and a Non-Template Control (NTC). Each square corresponds to a single real-time PCR reaction. Cq-values for each reaction are indicated by color; the corresponding color scale is presented in the legend on the right. A black square is considered as a negative result.

and 6.8 μM of probe or 33 μM of each primer and 10 μM of probe) with 3 μL 2X Assay loading reagent (Fluidigm). qPCR was performed in a BioMark 48.48DA (Fluidigm) combining 48 pre-amplified samples with 48 assays for 2304 individual and simultaneous qPCR reactions. The 48.48DA was primed in the IFC controller MX (Fluidigm) prior to loading of samples and assays. Sample mix (4.9 μL), and primer mix (4.9 μL) was dispensed into inlets on the 48.48DA, which was again placed in the IFC controller for loading and mixing of the 48 samples and 48 assays. After approximately 55 min the 48.48DA was ready for thermal cycling in the high-throughput qPCR instrument BioMark (Fluidigm) with the following cycling conditions: 15 min at 95°C, followed by 40 cycles at 94°C for 10 s, at 54°C for 30 s, and 72°C for 10 s. Non-template controls were included to control non-specific amplification and sample contamination. Specificity and sensitivity of all assays were tested against six virus isolates, representing targets for one or more of the different assays and thus the virus isolates functioned as both positive and negative controls for the individual primer and probe sets. Data (Cq-values and amplification curves) were acquired on the BioMark system and analyzed using the Fluidigm Real-Time PCR Analysis software 4.1.3 (Fluidigm).

Validation of Sensitivity of the qPCR Assays

To test and compare the performance and dynamic range of the qPCR assays on the Rotor-Gene Q platform and on the high-throughput qPCR BioMark platform, RNA 10-fold serial dilutions from six different swIAV isolates were tested on the Rotor-Gene Q, and the same RNA dilutions were cDNA synthesized and pre-amplified and then tested on the BioMark. Furthermore, 10-fold serial dilutions were made from the pre-amplified cDNA from the six swIAV isolates and these were only tested on the BioMark platform.

Verification of the Specificity of the swIAV 48.48DA

The performance of the swIAV 48.48DA was verified by testing 32 field samples (nasal swabs, oral fluid, and lung tissue samples) and 29 virus isolates (**Tables 1**, **2**). The full genome sequences were known for the virus isolates (Supplementary Table 1), while only the type of HA and NA genes were known for the field samples. The field samples have previously been tested and subtyped by an in-house multiplex RT-qPCR (modified from Henritzi et al., 2016) for diagnostic purposes.

Data Availability Statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

RESULTS

Specificity and Sensitivity of the qPCR Assays

RNA obtained from a panel of six IAVs of subtype H1, H3, N1, and N2 of avian, human, or porcine origin was used to evaluate the sensitivity and specificity of the different sets of primers and probes. The specificity of each assay was assessed from the Cq-value obtained from their respective target in relation to any cross reaction. For all qPCR assays, specific positive reactions were registered and no cross reactions were observed (**Figure 1**). The 18 selected assays discriminated correctly between the different linages of the HA gene ($H1_{av}$, $H1_{hu}$, $H1_{pdm}$, $H3_{hu}$, $H3_{sw}$) and NA gene ($N1_{av}$, $N1_{pdm}$, $N2_{sw}$, $N2_{hu}$). The qPCR assays specific for the internal genes discriminated in all cases between the pandemic and non-pandemic genes (**Figure 1**). Series of 10-fold diluted RNA of the six virus isolates were tested on the Rotor-Gene Q and on the swIAV 48.48DA to assess the relative analytical sensitivity of the qPCR assays. Comparisons of the Cq-values of the dilutions revealed that, in general, the dynamic range of the assays was 2–5 $\log_{10}$ for the swIAV 48.48DA and four-six $\log_{10}$ for the Rotor-Gene system (**Table 5**). For some of the assays the undiluted sample was not tested due to too small amount of available sample material. The dynamic range of the qPCR assays was generally 1–2 log higher using the Rotor-Gene Q compared to the swIAV 48.48DA. Ten-fold serial dilutions of the pre-amplified cDNA resulted in similar dynamic range and efficiency as the RNA dilutions for each of the qPCR assays (data not shown).

TABLE 5 | Relative sensitivity of qPCR assays on the Rotor-Gene Q platform and on the swIAV 48.48DA (BioMark platform).

	Assays											
Dilution	**$H1_{av}$**		**$H1_{hu}$**		**$H1_{pdm}$**		**$H3_{hu}$**		**$H3_{sw}$**		**M**	
	Rotor-Gene	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**
10	14.20	17.29*	15.57	17.40	14.58	13.44	-	17.71	13.93	14.22	-	11.85
10^{-1}	18.60	23.79	19.03	22.46	17.56	17.98	22.87	21.22	17.05	16.87	14.06	17.33
10^{-2}	22.69	28.12	22.66	26.03	20.79	19.98	25.81	23.78	20.44	19.43	17.00	22.01
10^{-3}	25.75	31.49	25.88	29.13	24.17	23.64	29.43	27.53	24.04	24.07	20.34	23.69
10^{-4}	28.86	neg	29.30	neg	27.20	28.88	32.70	neg	28.16	28.27	24.09	27.81
10^{-5}	32.66	neg	32.42	neg	30.97	neg	35.28	neg	neg	neg	27.53	29.45
10^{-6}	neg	neg	neg	neg	34.22	neg	neg	neg	neg	neg	29.96	neg
10^{-7}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Effectivity	0.89	0.80	0.98	0.80	1.01	0.88	1.07	1.04	0.91	1.01	1.02	0.84
Dilution	**$N1_{B1}$**		**$N1_{B2}$**		**$N1_{pdm}$**		**$N2_{B1}$**		**$N2_{B2}$**		**$N2_{hu}$**	
	Rotor-Gene	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**
10	18.09	15.15	19.14	17.21	18.04	16.24	14.54	15.53*	12.47	15.34	12.70	7.73
10^{-1}	22.05	19.69	22.42	21.55	21.01	20.73	17.15	21.35	16.20	21.08	14.71	12.81
10^{-2}	25.77	21.55	25.17	23.91	25.15	22.39	20.19	25.74	19.03	25.81	18.35	16.88
10^{-3}	29.50	25.57	28.76	27.14	28.75	26.79	23.36	29.32	22.00	28.78	22.28	18.95
10^{-4}	32.70	29.25	32.22	29.67	31.26	28.40	26.43	32.32	24.89	30.83	26.50	21.70
10^{-5}	36.33	neg	35.76	neg	neg	neg	30.93	neg	29.31	neg	30.25	23.58
10^{-6}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	34.42	neg
10^{-7}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Effectivity	0.89	1.01	1.00	1.02	0.96	1.07	1.04	0.89	1.04	0.82	0.89	1.11
Dilution	**$PB2_{pdm}$**		**$PB1_{pdm}$**		**PA_{pdm}**		**NP_{pdm}**		**NS_{pdm}**		**M_{pdm}**	
	Rotor-Gene	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**	**Rotor-Gene**	**BioMark**
10	15.32	13.79	-	19.67	-	18.49	-	-	-	16.45	13.81	12.58
10^{-1}	20.13	16.78	22.08	22.91	17.88	21.76	18.22	17.25	17.26	20.35	16.82	17.14
10^{-2}	25.27	19.31	24.62	25.85	22.12	24.67	21.59	20.97	20.30	23.26	20.62	20.16
10^{-3}	27.6	22.35	27.74	29.02	24.94	28.27	24.88	25.09	23.80	26.06	24.46	24.36
10^{-4}	29.95	26.87	31.53	32.14	28.19	32.56	29.11	27.68	27.16	28.18	27.93	28.07
10^{-5}	neg	29.49	34.65	neg	32.61	neg	32.18	neg	30.64	neg	30.53	neg
10^{-6}	neg	neg	37.43	neg	neg	neg	neg	neg	neg	neg	33.67	neg
10^{-7}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Effectivity	0.87	1.00	1.07	1.09	0.93	0.94	0.92	0.89	0.98	1.06	0.98	0.82

Validation of the swIAV 48.48DA Chip

In order to validate the performance of the swIAV 48.48DA for subtyping of swIAVs, a total of 29 well-characterized virus isolates and 32 field samples were tested. The subtype of the samples had previously been determined by either full genome sequencing or multiplex RT-qPCR and the results obtained by the swIAV 48.48DA were compared to these findings (**Tables 1**, **2**).

Of the 29 virus isolates, which have previously been full genome sequenced, 27 showed identical results when the subtyping was performed on the swIAV 48.48DA and by sequencing (**Table 2**). For each of the remaining two isolates there was a discrepancy for one of the genes. By full genome sequencing, the M gene of A/Swine/Denmark/4790-1/2015 had 93% identity with both pandemic and non-pandemic M genes of Danish swIAV strains (results not shown). The sample gave a positive signal for M_{pdm} on the swIAV 48.48DA despite that there were two mismatches in the primer and probe bindings regions and was by that defined as M_{pdm}. The NP gene of A/Swine/Denmark/03627-2/2015 was subtyped as being of non-pandemic origin by the swIAV 48.48DA, while based on the full genome sequence analysis the NP gene was found to be pandemic. The sequence analysis also revealed one mismatch in the binding site of the reverse primer and two mismatches between the probe binding sites for the NP_{pdm} assay. Thus, these

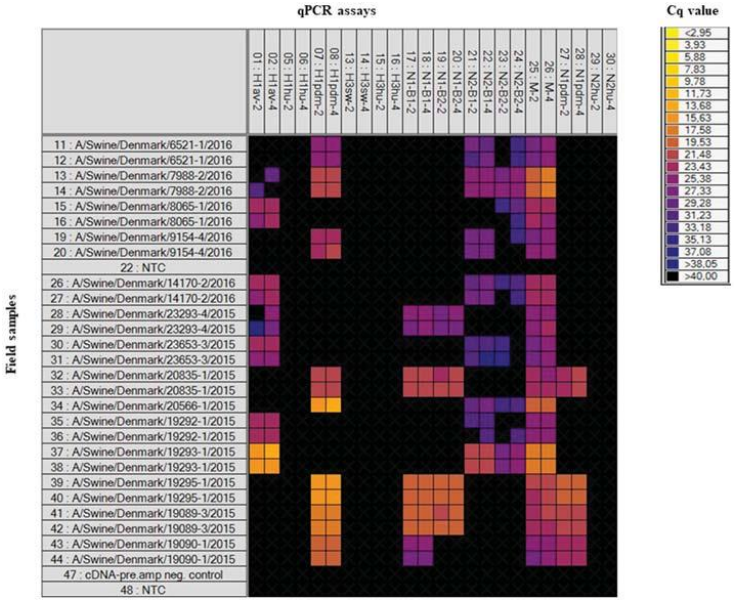

FIGURE 2 | Heat map showing the results of a subset of the tested field samples on the swIAV 48.48DA. Top: The HA, NA, and M specific assays in two different primer/probe concentrations (indicated by the numbers two or four). Left: number 11–44 (except for number 22) is the tested field samples in duplicates, number 47 is a negative control for the cDNA-pre.amp setup and number 22 and 48 is Non-Template Controls (NTC). Each square corresponds to a single real-time PCR reaction. Cq-values for each reaction are indicated by color; the corresponding color scale is presented in the legend on the right. A black square is considered as a negative result.

mutations could explain the discrepancy between the results obtained by sequencing and by test on the swIAV 48.48DA. Another sample, A/Swine/Denmark/ 20566-1/2015, was found to have the subtype $H1_{pdm}N2_{sw}$ with pandemic internal genes in both the full genome sequencing and when tested on the swIAV 48.48DA. However, this sample also tested positive in the assay specific for the $H1_{av}$ gene (**Table 2**). Retesting of the sample on the Rotor-Gene Q in the $H1_{av}$ and $H1_{pdm}$ assays confirmed these results, indicating that this sample contained two different viruses.

Field samples ($n = 32$), consisting of nasal swabs, lung tissue or oral fluid, were also analyzed on the swIAV 48.48DA (**Table 1**). These samples had previously been subtyped by an in-house multiplex RT-qPCR assay (modified from Henritzi et al., 2016), thus only the HA and NA genes were known for these samples. The heat map in **Figure 2** shows the results of a subset of the tested field samples in which the subtype for each of the sample was clarified based on the Cq-value and on the accuracy of the corresponding amplification curve. The swIAV 48.48DA and the multiplex RT-qPCR revealed the same HA type for 30 of the samples. However, none of the qPCR methods could define the HA subtype of sample A/Swine/Denmark/9079-2/2016. Furthermore, the HA subtype was not defined by the multiplex RT-qPCR for sample A/Swine/Denmark/6686-1/2015, but it was successfully determined using the swIAV 48.48DA. The sample A/Swine/Denmark/7988-2/2016 was found positive in both the $H1_{av}$ and $H1_{pdm}$ assay by the swIAV 48.48DA, but only positive for $H1_{pdm}$ in the multiplex RT-qPCR. Therefore, this sample was further tested in the $H1_{av}$ and $H1_{pdm}$ assays on the Rotor-Gene Q, where it was found positive in both assays indicating infection with two different viruses. For the NA assays, 29 of 32 samples were found to have the same NA linage by both qPCR typing methods. For the sample A/Swine/Denmark/6598-1/2016 no signal was obtained in any of the NA assays on the swIAV 48.48DA, while it was positive in the $N2_{B2}$ assay in the multiplex RT-qPCR. The sample was also tested in the N2 assay on the Rotor-Gene Q, where it was found to be weakly positive, with a Cq-value around 30. For the samples A/Swine/Denmark/14170-2/2016 and A/Swine/Denmark/8938-1/2015, no NA signal was obtained in the multiplex RT-qPCR, while the swIAV 48.48DA detected a signal in the $N2_{B1}$ and $N2_{B2}$ assays and in the $N1_{B2}$, respectively. The swIAV 48.48DA found sample A/Swine/Denmark/7961-7/2016 to be of both $N1_{pdm}$ and $N2_{sw}$ origin, while this sample was only positive in the $N1_{pdm}$ assay when using the multiplex RT-qPCR. Additional test on the Rotor-Gene Q found also this sample to be positive in the $N2_{B1}$ assay—again indicating that the samples contained two different viruses.

TABLE 6 | Comparison of the number of positive findings using the gold-standard test compared to the swIAV 48.48DA (BioMark) test (percentage in parentheses).

Genes	Gold-standard test	swIAV 48.48DA (BioMark)
$H1_{av}$	32/61 (52.5%)	35/61 (57.4%)
$H1_{pdm}$	26/61 (42.6%)	26/61 (42.6%)
$H3_{sw}$	0/61 (0%)	0/61 (0%)
$H3_{hu}$	1/61 (1.6%)	1/61 (1.6%)
N1	7/61 (11.5%)	8/61 (13.1%)
$N1_{pdm}$	14/61 (23.0%)	14/61 (23.0%)
N2	34/61 (55.7%)	35/61 (57.4%)
$N2_{hu}$	4/61 (6.6%)	4/61 (6.6%)
$PB2_{pdm}$	19/29 (65.5%)	19/29 (65.5%)
$PB1_{pdm}$	18/28 (64.3%)	19/29 (65.5%)
PA_{pdm}	19/29 (65.5%)	19/29 (65.5%)
M_{pdm}	20/29 (69.0%)	20/29 (69.0%)
NP_{pdm}	20/29 (69.0%)	19/29 (65.5%)
NS_{pdm}	19/29 (65.5%)	19/29 (65.5%)

In summary, when comparing the results for the swIAV 48.48DA with the sequencing and multiplex RT-qPCR results for the virus isolates and field samples, fully matching subtyping results (based on HA and NA genes) were obtained for 57 (29 virus isolates and 28 field samples) of 61 tested samples, and three of the 57 samples also showed an additional subtype in the analysis with the swIAV 48.48DA indicating a double infection. Furthermore, when comparing the number of positive findings in the gold-standard tests (sequencing and multiplex RT-qPCR) with the swIAV 48.48DA test an agreement was observed for nine of the tested genes, while a difference between 1.2 and 4.9 % was observed for the rest of the genes (**Table 6**).

DISCUSSION

The BioMark high-throughput qPCR protocol for detection and expanded subtyping of influenza virus in pigs described in the present paper proved to be as specific and sensitive as standard state-of the art diagnostic methods based on "conventional" qPCR and sequencing. This new approach makes it possible to combine multiple assays and samples and run them simultaneously. It requires less labor and pipetting, leading to an economical benefit. Another benefit is the use of nanolitre volume chambers in the DA, in contrast to conventional qPCR that uses microliter, thereby decreasing the use of expensive reagents. The BioMark high-throughput qPCR system has for years been widely used in research studies i.e., for the study of innate immune response to pathogens (Skovgaard et al., 2013). More recently, high-throughput qPCR protocols using the BioMark platform have also been designed as surveillance tools for tick-borne diseases and for food- and waterborne pathogens (Ishii et al., 2013; Michelet et al., 2014). Similar to the present study, Ishii et al. (2013) found the system to offer highly sensitive and specific simultaneous quantification of multiple food-and waterborne pathogens in multiple samples (Ishii et al., 2013). The platform is a flexible tool because it is easy to modify the assay panel by adding or removing primers or probes when new pathogens or new variants emerge (Ishii et al., 2013; Michelet et al., 2014).

To our knowledge this is the first paper describing the use of the BioMark high-throughput qPCR platform for detection and subtyping of influenza viruses. In general, there was a high degree of agreement for the results provided by multiplex RT-qPCR or sequencing and the results generated by the swIAV 48.48DA. For a few of the tested samples, there was a discrepancy. These differences could be explained by either co-infection with two viruses or by mismatches in the primer/probe binding regions. Thus, imperfect match between the target sequence and the primer and/or probe sequences can result in a false-negative signal even though the sample is positive for swIAV. This emphasizes that the swIAV 48.48DA or multiplex RT-qPCR protocols cannot stand alone as a subtyping method, but has to be combined with a continuous surveillance by sequencing of circulating swIAV isolates. Due to the high mutation- and reassortant rate of IAVs (Simon et al., 2014) it is important to do continuous sequencing of selected isolates because changes will occur over time and it is necessary to adjust the PCR assays accordingly. Sequencing is a very informative tool and it can contribute with indispensable information about evolutionary relationships based on similarities and differences between the sequences. However, since the number of isolates that can be sequenced is limited by practical and economic reasons, the swIAV 48.48DA provides an excellent screening tool for selection of atypical isolates for downstream characterization by sequencing.

Pre-amplification of the RNA samples was needed because of the very small sample volumes (<10 nL; Korenková et al., 2015) in the reaction chambers. This is in accordance with recommendations from the supplier and previous studies using the BioMark protocols for the detection of i.e., water-borne pathogens (Ishii et al., 2013). The supplier of the BioMark platform recommends performing the cDNA synthesis and pre-amplification as two separate steps. However, we managed to change this into a one-step procedure by combining the cDNA synthesis and pre-amplification, which further reduced the analysis costs and the number of handling steps. A benefit of this alteration is also the reduced risk of contamination due the fewer handling steps. The swIAV 48.48DA was tested against a panel of representative virus isolates in order to assess the sensitivity and specificity. All the assays had an acceptable PCR efficiency between 80 and 110%. Comparison of the assay performance on the two qPCR platforms; Rotor-Gene Q and BioMark, revealed only a minor difference in the dynamic range and efficiency for all the assays. For a majority of the qPCRs, the dynamic range was one-two $\log_{10}$ higher on the Rotor-Gene Q platform compared to the BioMark. This might be a result of the considerable lower reaction volume in the 48.48DA (<10 nL) compared to the tubes (25 µl) of the Rotor-Gene Q. No cross reactions were observed for any of the assays on the swIAV 48.48DA, which testifies a high specificity. To test the specificity in more detail, virus isolates and field samples, which have previously been subtyped by sequencing or multiplex RT-qPCR,

were tested on the swIAV 48.48DA. Again no cross reactions were observed and the three field samples, which failed to provide a signal in the HA or NA analysis in the multiplex RT-qPCR test, were subtyped by the swIAV 48.48DA. This difference can be explained by the ability of the swIAV 48.48DA to subtype weakly positive samples (Cq-value of 30 or above in the M qPCR assay) which cannot be subtyped using the standard multiplex RT-qPCR protocol. The improved sensitivity of the swIAV 48.48DA is related to the 24 pre-amplification cycles used prior to the PCR step.

The heat map generated by the Fluidigm Real-Time PCR Analysis software illustrates the raw Cq-values for each reaction, which makes is feasible to quickly evaluate which subtype the individual samples have (**Figure 2**). Using the swIAV 48.48DA for the subtyping of swIAVs in surveillance programs, will make the analysis more simple compared to the traditional subtyping methods and it will give a more detailed subtyping of the samples since the internal genes are included in the analysis.

In summary, the use of the swIAV 48.48DA will allow future subtyping of many more influenza virus isolates for the same resources and by that contribute to a more sensitive surveillance program and provide the basis for an improved early detection of new virus re-assortments and variants. The high sensitivity, specificity and robustness of the test system may also provide an opportunity for development of other similar chips i.e., for the surveillance and diagnose of other veterinary pathogens. Work is in progress on the development of a 48.48DA containing all important swine pathogens for the use in future surveillance and diagnostic programs in Danish swine herds.

AUTHOR CONTRIBUTIONS

LL, JK, CH, KS, and NG contributed to the experimental design of the study. qPCR assays, which have been designed in the present study, have been designed by JK, TH, SB, and NG. PCR analyses were conducted and interpreted by NG. The main manuscript was initially drafted by NG and LL has contributed to the manuscript preparation, while all authors participated in proofreading of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00165/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Manuscript II

Development of a high-throughput real-time PCR system for diagnosis of enzootic pathogens in pigs

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Abstract

Respiratory and intestinal diseases in pigs can have significant negative influence on the productivity and animal welfare. The diagnostic laboratory at the National Veterinary Institute in Denmark analyses thousands of samples each year for the presence of porcine pathogens. Presently, a wide range of different quantitative real-time polymerase chain reaction (qPCR) assays are used for the pathogen detection and the PCR analyses are performed on traditional real-time PCR platforms, in which a limited number of samples can be analysed per day due to limitations in equipment and personnel. To mitigate these restrictions, the qPCR assays have been optimised for the high-throughput qPCR BioMark platform (Fluidigm). The BioMark platform uses an integrated fluidic circuit (IFC) technology to prepare and perform qPCR reactions in nanoliter volumes and it is capable of performing up to 9,216 parallel reactions in a single run. Using this platform, we have developed a high-throughput diagnostic system, which can be used for simultaneous examination of 48 samples with detection specificity for 17 selected respiratory and enteric viral and bacterial pathogens of high importance to the Danish pig production. qPCR assays were validated and optimized to run under the same reaction conditions using a BioMark 48.48 dynamic array (48.48DA) IFC chip and the sensitivity and specificity were assessed by testing known positive samples. The results revealed a performance of the diagnostic 48.48DA similar to traditional qPCR analysis, and the specificity of the diagnostic 48.48DA was high. Application of the high-throughput platform results in a significant reduction in cost and working hours and provides production herds with a new innovative diagnostic service with the potential to facilitate the optimal choice of disease control strategies such as vaccination and treatment.

Keywords

Respiratory porcine pathogens, enteric porcine pathogens, diagnostics, high-throughput, real-time PCR.

Introduction

Respiratory and intestinal diseases are of major importance in the commercial pig production throughout the world as these diseases can result in major economic losses due to reduced productivity, decreased animal welfare, increased mortality or morbidity and increased use of antibiotics. The cause of disease can either be single- or multifactorial. The prevalence and combination of pathogens can fluctuate between countries and herds, and the composition of pathogens can change over time due to e.g. change in management, environmental changes, season and stage of infection (Hansen et al., 2010; Heo et al., 2013; Hernandez-Garcia et al., 2017; Opriessnig et al., 2011; Stärk, 2000).

Pathogens involved in the porcine respiratory disease complex (PRDC) include bacteria such as *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Pasteurella multocida* and *Streptococcus suis*. Viral agents of PRDC includes porcine circovirus type 2 (PCV2), porcine cytomegalovirus (PCMV), porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza A virus (swIAV). Many of the respiratory pathogens are ubiquitous and it is not uncommon for multiple pathogens to circulate in a herd at any given time without causing disease (Brockmeier et al., 2002; Harms et al., 2002; Kim et al., 2003; Opriessnig et al., 2011; Phan et al., 2016; Thacker et al., 2001). However, the respiratory pathogens may also cause clinical symptoms such as depression, anorexia, fever, slight nasal and ocular discharge, cough, slight hyperpnoea and even fatal pneumonia (Janke, 1995; Opriessnig et al., 2011; Thacker, 2001).

Intestinal diseases can be induced by a number of different viruses, bacteria and parasites, and are also considered as a multifactorial problem. Diarrhoea together with dehydration, reduced feed intake, reduced growth and reduced nutrient digestibility are symptoms associated with intestinal disorders (Fouhse et al., 2016; Heo et al., 2013; Jacobson et al., 2003). Some of the most frequently found bacteria associated with intestinal disease in Danish pigs are *Lawsonia intracellularis*, *Brachyspira pilosicoli* and *Escherichia coli* fimbria types F4 and F18 (Jacobson et al., 2003; Pedersen et al., 2014; Weber et al., 2015). Viruses such as rotavirus A and PCV2 may also contribute to enteric disease. Rotavirus A is a known diarrhoea causing agent in pigs (Bohl et al., 1978), while PCV2 has not been proven to be a primary cause of diarrhoea in pigs but systemic PCV2 may, however, indirectly contribute to enteric diseases due to its immunosuppressive effect (Jensen et al., 2006; Johansen et al., 2013; Segalés, 2012). Coronaviruses such as transmissible gastroenteritis virus, porcine deltacoronavirus and porcine epidemic diarrhoea virus can also induce

enteric diseases, but Denmark and many other European countries are free of these viruses (Pensaert and Martelli, 2016).

The choice of suitable control measures such as vaccination and antibiotic treatment are often based on clinical manifestations without support from laboratory investigations. This may lead to overuse of antibiotics and suboptimal vaccination programs. The diagnostic laboratory of the National Veterinary Institute, Technical University of Denmark, analyses thousands of samples each year for the presence of pathogens in Danish pigs. Today, a wide range of different quantitative real-time polymerase chain reaction (qPCR) assays are used for the pathogen detection and the analyses are performed on traditional or so-called low-throughput qPCR platforms. A major bottleneck of these platforms is the limited number of different targets that can be tested at the same time, and furthermore, these qPCR analyses are expensive and resource demanding, both when it comes to reagents and working time (wage cost). Thus, there is a need for the development of faster and cheaper diagnostic systems.

The aim of the present study was to develop a high-throughput qPCR detection system capable of detecting significant porcine viruses and bacteria in the same setup. For this, the microfluidic high-throughput qPCR BioMark platform (Fluidigm, South San Francisco, USA), which can perform parallel qPCRs, was utilized. The BioMark dynamic array (DA) system (Fluidigm) is able to combine e.g. 48 samples with 48 assays or 96 samples with 96 assays or 192 samples with 24 assays in a combinatorial manner inside the integrated fluidic circuit (IFC) resulting in either 2,304, 9,216 or 4,608 individual reactions in a single run (Liu et al., 2003). The present study describes the design, optimization and validation of a diagnostic 48.48DA; a setup consisting of 21 qPCR assays targeting 17 selected respiratory and enteric viral and bacterial pathogens of high importance in the Danish swine industry.

Materials and methods

Samples

Known positive samples (controls) for each of the 17 selected pathogens were used for optimization and initial validation of the diagnostic 48.48DA. The positive controls consisted of cell culture lysates (viruses), pure bacterial cultures, vaccine strains and plasmids. Furthermore, 91 field samples (oral fluid, faecal sock, lung tissue and liver tissue samples), from routine submissions to the National Veterinary Institute, were used for validation of the diagnostic 48.48DA. These samples came from pig herds with a history of respiratory or intestinal disease and had been tested

in the routine diagnostic setting at the Danish National Veterinary Institute for presence of pathogen(s).

RNA and DNA extraction

Viral RNA was extracted from cultured viruses, oral fluid, lung tissue or nasal swab samples by the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions using the large sample protocol version 2 on the QIAcube (QIAGEN) extraction robot. Cell culture supernatant, oral fluid and nasal swab samples were prepared by mixing 200 µL material with 400 µL RLT buffer containing β-mercaptoethanol (Sigma-Aldrich). Lung tissue samples were prepared by homogenization of 70 mg lung tissue in 1400 µL RLT buffer containing β-mercaptoethanol (Sigma-Aldrich) on a TissueLyser II (QIAGEN) at 30 Hz in 3 min. The homogenate was centrifuged for 3 min at 12,000 *g*, and RNA was extracted from 600 µL of the supernatant. Viral RNA was eluted in 60 µL RNase-free water and stored at -80°C.

Viral and bacterial DNA were extracted from oral fluid, lung tissue or liver tissue samples by the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions using the tissue standard protocol version 1 on the QIAcube (QIAGEN) extraction robot. Oral fluid samples were prepared by centrifugation of 200 µL sample at 1,000 rpm for 3 min, followed by a tenfold dilution in nuclease free water. Lung and liver tissue samples were prepared by homogenization of 180 mg lung tissue in 1300 µL ATL buffer on a TissueLyserII (QIAGEN) in 3 min. The homogenate was centrifuged for 3 min at 12,000 *g*, and 200 µl of the supernatant was mixed with 20 µl Proteinase K and incubated for 10 min at 56°C. DNA was eluted in 200 µL Buffer AE and stored at -20°C.

10% faeces in PBS samples were extracted using the QIAsymphony SP system (QIAGEN) extraction robot and QIAsymphony DSP virus/pathogen mini kit (QIAGEN) following the manufacturer's instructions. The protocol was Complex200_V5_DSP with an elution volume of 110 µL. Before extraction, one 5 mm steel bead was added to each sample and the samples were homogenized in a Tissuelyser II (QIAGEN) for 20 sec at 15 Hz. The homogenate was then centrifuged for 90 sec at 10,000 rpm and the 350 µL of the supernatant was used for nucleic acid extraction. The nucleic acid extractions were stored at -80°C until further analysis.

Primer and probe design

The diagnostic 48.48DA was designed to include 21 qPCR assays primarily targeting respiratory and enteric viral and bacterial pathogens using the sets of primers and probes listed in Table 1 and 2. The primer and probe sequences were either from previously published assays or designed for the present study. Some of the published primer and probe sequences were slightly modified to improve their specificities, and the fluorophore and/or quencher were changed for some of the assays as well. These modifications are highlighted in bold in Table 1 and 2. Furthermore, for the PCV2 and PPV assays the detection chemistry was changed from Primer Probe Energy transfer to dual labelled probe chemistry to match the remaining assays. New primer and probe sequences were designed based on alignments comprising full-length or nearly full-length sequences of target gene for the selected pathogen. Sequences used for the alignments were retrieved from GenBank (NCBI Resource Coordinators, 2017) and aligned using CLC Main Workbench version 7.7.3 (https://www.qiagenbioinformatics.com/). Oligonucleotide specificity was tested *in silico* using BLAST search (Altschul et al., 1990), while melting temperature and basic properties of the oligonucleotides were approximated using OligoCalc (Kibbe, 2007). The primer and probe sequences were purchased from Eurofins Genomics (Ebersberg, Germany) and stored at -20°C. The sensitivity and specificity of each qPCR assay were validated based on a tenfold serial dilution range of positive controls (Table 3 and 4) on the Rotor-Gene Q qPCR system (QIAGEN, Hilden, Germany).

Traditional qPCR on Rotor-Gene Q

Assays targeting RNA viruses

For the RNA targets, different master mixes and PCR conditions were tested during the initial validations. The RT-qPCR assay specific for Rotavirus A was performed in a final volume of 15 µl using AgPath-ID one-step RT-PCR reagents kit (Applied Biosystems, Foster City, USA) with 3 µL RNA. 7.5 µL RT-PCR buffer (2X) was mixed with 0.12 µL of each primer (50 µM), 0.18 µL probe (10 µM), 0.6 µL RT-PCR enzyme mix (25X) and nuclease free water. The thermal cycling conditions were 45°C for 10 min, 95°C for 10 min followed by 48 cycles at 95°C for 15 sec and 60°C for 45 sec. The fluorescence signal was acquired at the 60°C step in the Green channel (470-510 nm).

The RT-qPCR assay specific for swIAV was performed in a final volume of 25 µL using QIAGEN One-Step RT-PCR kit (QIAGEN), with 5 µL QIAGEN One-Step RT-PCR buffer (5X), 1 µL dNTP mix (10 mM), 1.25 µL MgCl2 (25 mM), 1 µL of each primer (100 µM), 0.25 µL probe (30 µM), 1 µL QIAGEN enzyme mix, 2 µL RNA and nuclease free water. Thermal cycling conditions were: 50°C for 30 min, 95°C for 15 min followed by 40 cycles at 94°C for 10 sec, 54°C for 30 sec and 72°C for 10 sec. The fluorescence signal was acquired at the 54°C step in the Green channel (470-510 nm).

The PRRSV-specific RT-PCR assays were performed in a final volume of 25 µL using QIAGEN One-Step RT-PCR kit (QIAGEN) with 2 µL RNA. The mixes consisted of 5 µL QIAGEN One-Step RT-PCR buffer (5X), 1 µL dNTP mix (10 mM each), 0.75 µL of each primer (10 µM), 0.50 µL probe (10 µM), 1 µL QIAGEN enzyme mix and nuclease free water. The thermal cycling conditions were: 50°C for 30 min, 95°C for 15 min, followed by 45 cycles at 94°C for 15 sec, 60°C for 60 sec and 72°C for 10 sec. The fluorescence signal was acquired at the 60°C step in the Green channel (470-510 nm).
Data obtained from each of the above mentioned assays were analysed with the Rotor-Gene Q Series Software 2.3.1 (QIAGEN) with the following parameter adjustments: dynamic tube normalization, on; noise slope correction, on; ignore first cycle; outlier removal, 10%; quantification cycle (Cq) threshold fixed, 0.01. All reactions were run in duplicates together with positive and negative controls.

Assays targeting DNA viruses and bacteria
For the DNA targets (except for PCV3), the qPCR assays were performed in a final volume of 25 µl using the JumpStart Taq ReadyMix (Sigma-Aldrich, St. Louis, USA) with 3 µL of DNA. The PCR mixes for *L. intracellularis, B. pilosicoli, E. coli* F4 and *E. coli* F18 were prepared as previously described by Ståhl *et al.*, 2011 (Ståhl et al., 2011), while the PCR mixes for the remaining DNA targets contained 12.5 µL JumpStart Taq ReadyMix (2X), 0.75 µL of each primer (10 µM), 0.21 µL probe (30 µM), 3.5 µL $MgCl_2$ (25 mM) and nuclease free water. All amplifications were run at the same cycling conditions: 94°C for 2 min followed by 40 cycles of 94°C for 15 sec and 60°C for 60 sec. The PCV3 assay was performed in a final volume of 25 µL with 2.5 µL AmpliTaq Gold buffer (10x) (Applied Biosystems), 0.5 µL dNTPs (10 mM), 2.5 µL $MgCl_2$ (25 mM), 0.75 µL of each primer (10 µM), 0.5 µM probe (10 µM), 0.25 µL AmpliTaq Gold DNA polymerase [5 U/µL]

(Applied Biosystems), nuclease free water and 3 µL DNA. The cycling conditions were: 94°C for 10 min followed by 45 cycles at 94°C for 15 sec and 60°C for 60 sec, where the fluorescence signal was acquired at the 60°C step in the Green channel (470-510 nm). Data obtained from each of the above mentioned assays were analysed with the Rotor-Gene Q Series Software 2.3.1 (QIAGEN) with the following parameter adjustments: dynamic tube normalization, on; noise slope correction, on; ignore first cycle; outlier removal, 10%; threshold fixed, 0.01. All reactions were run in duplicates with positive and negative controls.

Reverse transcription and pre-amplification prior to high-throughput qPCR

A primer mix containing all the different set of primers (20 µM) listed in Table 1 and 2 was prepared.

For the RNA targets, reverse transcription and pre-amplification was performed in a final volume of 25 µL using the AgPath-ID one-step RT-PCR reagents kit (Applied Biosystems, Foster city, USA), in which 12.5 µL of 2X RT-PCR buffer was mixed with 1 µL 50 µM random hexamer (Invitrogen, Carlsbad, California, USA), 1.25 µL 20 µM primer mix, 1 µL 25X RT-PCR enzyme mix, 6.25 µL nuclease-free water and 3 µL RNA. One-tube combined reverse transcription and pre-amplification was performed on a T3 Thermocycler (Biometra, Fredensborg, Denmark) with the following thermal cycling conditions: 20 min at 45°C, 10 min at 95°C followed by 24 cycles at 94°C for 15 sec and 60°C for 45 sec. The pre-amplified cDNA was stored at -20°C.

For pre-amplification of the DNA targets, the TaqMan PreAmp master mix (Applied Biosystems) was used following the manufacturer's instructions. The reaction was performed in a final volume of 10 µL containing 5 µL TaqMan PreAmp Master mix, 2.5 µL 20 µM primer mix and 2.5 µL DNA. Pre-amplification was performed on T3 Thermocycler (Biometra) with the following thermal cycling conditions: 95°C for 10 min, followed by 14 cycles at 95°C for 15 sec and 60°C for 4 min. The pre-amplified DNA was stored at -20°C.

High-throughput qPCR

For high-throughput qPCR amplification, the BioMark 48.48DA IFC chip (Fluidigm, South San Francisco, USA) was used. A 6 µL sample mix containing 3 µL TaqMan Gene Expression Master mix (Applied Biosystems), 0.3 µL 20X Sample Loading Reagent (Fluidigm) and 2.7 µL pre-amplified sample was prepared for each sample. A primer/probe stock was prepared for each assay and 3 µL of the stocks was mixed with 3 µL 2X Assay Loading Reagent (Fluidigm) to make assay

mixes (final concentration: 16 µM primers and 5 µM probe). Prior to loading of the sample and assay mixes, the 48.48DA was primed in the IFC controller MX (Fluidigm). 4.9 µL sample mix and 4.9 µL assay mix, for each of the samples and assays, were dispensed into the sample and assay inlets, respectively, on the 48.48DA. The 48.48DA was placed in the IFC controller MX for loading and mixing. After approximately 55 min, the 48.48DA was ready for thermal cycling in the high-throughput qPCR instrument BioMark with the following cycling conditions: 94°C for 2 min, followed by 40 cycles of 94°C for 15 sec and 60°C for 60 s. Assays were performed in duplicates and samples were performed in single reactions. Three positive controls, containing a mix of all the positive controls included in the screening, two non-template controls (nuclease-free water, Amresco), a non-template cDNA/pre-amplification and a non-template pre-amplification controls (nuclease-free water, Amresco) were included in each 48.48DA chip as controls for non-specific amplification and sample contamination.

Data, including Cq values and amplification curves, were acquired on the BioMark system and analysed using the Fluidigm Real-Time PCR Analysis software 4.1.3 (Fluidigm). The amplification efficiency was calculated for each qPCR assay based on the slope of the standard curve, as previously described (Bustin et al., 2009).

Validation of the diagnostic 48.48DA

The sensitivity and specificity of the qPCR assays was validated by running tenfold serial dilution for each of the positive control samples (Table 1 and 2) on the diagnostic 48.48DA. The performance of the diagnostic 48.48DA was validated by testing 91 field samples (DNA/RNA extracted from oral fluid, faecal sock, lung tissue and liver tissue samples), which previously have been tested by RT-qPCR or/and qPCR on the Rotor-Gene Q platform in the diagnostic laboratory at the National Veterinary Institute, Denmark.

Statistical analysis

The correlation between the high-throughput qPCR BioMark platform and the Rotor-Gene Q platform was investigated using Pearson correlation coefficient (PCC) and the associated p-value (Benesty et al., 2009). The analyses were performed using the analysis tool Regression Statistics in Microsoft Excel 2010.

Results

Specificity of the qPCR assays

Primer and probe sequences were designed to specifically detect seven viruses and ten bacteria species (Table 1 and 2). Viral RNA and DNA and bacterial DNA extracted from known positive samples were used as positive controls to evaluate the specificity of the different qPCR assays included on the diagnostic 48.48DA. Initially, the positive controls were run on the diagnostic 48.48DA without pre-amplification resulting in a lack of detection by the BioMark system for the majority of the samples. Consequently, an initial step of pre-amplification was added to the setup, which enabled the detection of all positive controls. The specificity of each qPCR assay was assessed from the Cq value and the corresponding amplification curve obtained from their respective positive control. For all the qPCR assays, specific positive reactions were registered, however, the assay specific for PPV cross reacted with the PPRSV type 1 positive control (Figure 1). Theoretically, this cross reaction could be due to lack of specificity or co-infection of the sample, but since the positive control for PRRSV type 1 was a vaccine strain, the cross reaction may be explained by the lack of specificity.

Sensitivity of the qPCR assays

The PCR efficiencies for the Rotor-Gene Q platform and the BioMark platform ranged from 89-107% and 85-110%, respectively (Table 3). To evaluate the sensitivity of the qPCR assays, series of tenfold diluted RNA and DNA of the positive controls were initially tested on the Rotor-Gene Q platform. Hereafter, the same dilutions of RNA and DNA were reverse transcribed/pre-amplified and pre-amplified, respectively, and tested on the BioMark platform. Comparison of the standard curves generated from the two platforms revealed that the dynamic range of the qPCR assays was either identical or had one $\log_{10}$ difference (Table 3), except for the assay specific for *A. pleuropneumoniae*. For *A. pleuropneumoniae*, the undiluted and the first diluted sample were excluded from the calculation of the effectivity (BioMark platform) due to too low Cq values. This exclusion resulted in a shorter dynamic range compared to the dynamic range for the dilution series analysed by the Rotor-Gene Q platform. For few of the assays the undiluted sample was not tested due to insufficient amount of available sample material.

Test of field samples on the diagnostic 48.48DA

In order to test the performance of the diagnostic 48.48DA and to validate the system, 91 field samples (oral fluid, faecal sock, lung tissue and liver tissue samples), which had previously been analysed by RT-qPCR/qPCR on the Rotor-Gene Q platform in the routine diagnostic laboratory at the National Veterinary Institute, were tested on the diagnostic 48.48DA and the results were compared. In general, the Cq values obtained by the diagnostic 48.48DA was lower than the Cq values obtained at Rotor-Gene Q platform, which is expected due to the additional pre-amplification step. Thirty-six faecal sock samples, previously tested for *L. intracellularis*, *B. pilosicoli* and *E. coli* F4 and *E. coli* F18 using the Rotor-Gene Q platform, were tested on the diagnostic 48.48DA. Almost identical results were achieved for the faecal sock samples on the two platforms and thus, the correlation between the two platforms was found to be significant for all four assays ($p < 0.05$) (Table 4). However, the outcome for four of the samples differed (marked with red in Table 4). Three of these samples were found to be positive in one assay in the Rotor-Gene Q test, but negative in the same assay on the diagnostic 48.48DA, while the last sample was found to be positive in the diagnostic 48.48DA test, but negative in the Rotor-Gene Q test.

Fourteen oral fluid samples, of which five of the samples were positive for PCV2 and nine of the samples were positive for swIAV on the Rotor-Gene Q platform, were also tested on the diagnostic 48.48DA. Here, the same five and nine samples were tested positive for PCV2 and swIAV, respectively (data not shown). Furthermore, 38 lung tissue samples, which had previously been analysed for other respiratory pathogens were also tested on the diagnostic 48.48DA. Comparisons of the results obtained by the two qPCR platforms are listed in Table 5. In general, the two platforms gave similar results, however, for the *P. multocida* analysis, the results for seven of the samples did not match. Six of these samples were found to be either negative or only slightly positive (denoted by low in table 5) in the analysis performed by the Rotor-Gene Q platform. The diagnostic 48.48DA analysis found the samples that were negative by the Rotor-Gene analysis to be positive, but with a relative high Cq value, and it found the slightly positive samples to be negative. The last sample (1991-3) of the seven was found to be present massively positive (Cq value < 25), but was negative on the diagnostic 48.48DA. Two differences were observed for a negative and a slightly positive *M. hyorhinis* sample. Furthermore, of the two PRRSV type 2 positive samples (455-5 and 455-6) the diagnostic 48.48DA analysis only found one (455-5) of these samples to be positive. However, the Cq value for sample 455-6 was 31.2, and this relative high value can be the reason why the sample was not detected by the diagnostic 48.48DA. The results obtained for the

qPCR assays specific for *A. pleuropneumoniae* and *M. hyopneumoniae* were 100% identical for the two platforms. In addition to the respiratory and enteric samples, three foetal liver tissue samples, which had been tested positive for PPV were also tested on the diagnostic 48.48DA and was found to be highly positive (Cq 3-5) (data not shown). The respiratory field samples (lung tissue samples) were also tested on the diagnostic 48.48DA for PCMV, PCV2, *S. suis* type 2, *B. bronchiseptica* and PCV3, which are not a part of the standard routine diagnostic testing scheme at the National Veterinary Institute (Table 6). The results revealed that PCMV and *S. suis* type 2 were highly prevalent. PCMV was detected with both low and high Cq values while *S. suis* type 2 was detected with mostly high Cq values. The remaining tested pathogens were sporadically detected with both low and high Cq values. However, since these samples were not tested using the Rotor-Gene Q platform no conclusion could be made in regards to sensitivity and specificity of these assays.

Discussion

To our knowledge, this is the first paper describing the use of the high-throughput qPCR BioMark platform as a diagnostic tool for detection of viral and bacterial pathogens in production animals. The high-throughput qPCR protocol for detection of respiratory and enteric viral and bacterial pathogens proved to be as specific and sensitive detection method as traditional qPCR. This new approach enables the combination of multiple assays and multiple samples run simultaneously in nanoliter volumes. This method requires less labour and reagent volumes compared to traditional qPCR methods, which require microliter volumes thus increasing the cost of analysis due to the use of higher amounts of expensive reagents. Furthermore, more working hours for set up and data analysis of the analyses are required. Recently, we designed a 48.48DA containing qPCR assays, which can differentiate between the different swIAV subtypes, circulating in European pigs (Goecke et al., 2018). The BioMark system has also been used as a screening and detection tool for food- and waterborne pathogens and for tick-borne diseases (Ishii et al., 2013; Michelet et al., 2014). These studies found the system to offer sensitive as well as specific detection of miscellaneous pathogens. The BioMark system is a flexible tool, allowing easy modification of the assay panel by adding or removing assays, provided that the assays have been optimized to the PCR conditions chosen for the given setup (Ishii et al., 2013; Michelet et al., 2014). This is a great advantage for a screening and detection tool, since it eases preparedness to new pathogens or new variants emerge.

The aim of the present study was to develop a diagnostic tool, which was specific for selected respiratory and enteric viral and bacterial pathogens of high importance to the swine industry in Denmark. The validation of the diagnostic 48.48DA with positive controls showed that pre-amplification of the samples was needed due to the very small reaction volumes (Korenková et al., 2015). The need for pre-amplification is in accordance with recommendations from the supplier and other studies using the BioMark for detection of pathogens (Ishii et al., 2013; Michelet et al., 2014; Spurgeon et al., 2008). In the present study, the pre-amplification of the target RNA and DNA was performed in two different setups. The pre-amplification of the DNA targets was performed according to supplier recommendations, while for the RNA targets, the cDNA synthesis and pre-amplification setup was optimised to a one-step procedure instead of the two-step procedure, as described previously (Goecke et al., 2018).

The performance of the qPCR assays specific for the selected pathogens was initially evaluated on positive controls using both the high-throughput and traditional qPCR platforms. All the qPCR assays had an acceptable PCR efficiency between 85-110% on the two qPCR platforms and comparison of the assay performances revealed only a minor difference in the dynamic range and efficiency for all the assays. The dynamic range was either the same or had one $\log_{10}$ difference between the two platforms, while the assay specific for *A. pleuropneumoniae* was an exception. Here the two first samples (10^0 and 10^{-1}) were excluded from the dilution series analysed by the BioMark platform due to too low Cq values. As observed for *A. pleuropneumoniae*, very positive samples can result in false negative results in the BioMark analysis which can be due to an inhibition of the system. In cases where a sample has a very low Cq value a light yellow colour will appear in the heat map indicating a positive sample, however, the amplification curve might not have the right shape, which can question the result. In such cases, a dilution of the sample will be necessary.

To evaluate the performance of the diagnostic 48.48DA, field samples which have previously been tested in the routine veterinary diagnostic laboratory by qPCRs were tested on the diagnostic 48.48DA. In general, there was a high degree of agreement for the results provided by the diagnostic laboratory and the results generated by the diagnostic 48.48DA. For a few of the tested faecal sock samples, there was a discrepancy between the two platforms, but the Cq value for the affected samples was relative high and therefore represent border line samples. However, a significant correlation between the two qPCR platforms was found for the four assays. The results obtained with the diagnostic 48.48DA for the oral fluid, lung tissue and liver tissue samples were

also consistent with the findings from the Rotor-Gene Q analyses with few exceptions. The assay specific for *P. multocida* was the assay, showing most discrepancies, but again these samples, except for one, were only weakly positive.

The use of the diagnostic 48.48DA for identification of respiratory and enteric viral and bacterial porcine pathogens provides new possibilities for veterinary diagnostics. The advantage of offering diagnostics for all relevant pathogens causing respiratory and intestinal diseases diminish the risk of not detecting the relevant pathogens in each specific case. The majority of the included pathogens were chosen based on their high prevalence in Danish pigs. However, additionally the diagnostic 48.48DA also includes test for the newly described virus PCV3, which was firstly discovered in 2016 in USA (Phan et al., 2016) and it has subsequently also been detected in Asia and Europe including Denmark (Franzo et al., 2018; Ku et al., 2017; Stadejek et al., 2017b).

In conclusion, we have developed a sensitive and specific diagnostic approach for simultaneous detection of multiple respiratory and enteric viral and bacterial porcine pathogens by using a microfluidic high-throughput qPCR platform. This new screening and detection approach is a powerful tool with the capacity of analysing a large number of samples in a large number of qPCR assays in the same analysis. Furthermore, it is easy to modify the assay panel by adding or removing assays. Thereby, this tool can easily be adapted to new situations in which e.g. new pathogens or new variants emerge. With this diagnostic tool it is possible to offer diagnostic services with reduced costs and turnover time, which may facilitate the correct choice of disease control strategies such as vaccines and promote a reduction in medicine use. Internal calculations reveal that full implementation and automatization of the procedure can result in an analysis cost per sample less than 5% of the cost of traditional qPCR, and thereby the assay have great potentials for future disease surveillance of pathogens in production herds.

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Author Contributions Statement

LL, JK, CH, KS and NG contributed to the experimental design of the study. Design and modification of the qPCR assays and the PCR analyses were done by NG. The manuscript was written by NG and LL has contributed to the manuscript preparation, while all authors participated in proofreading of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare no competing interests.

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Table 1 Primer and probe sequences for virus detection. Modifications of published sequences are in bold

Pathogen - Virus	Target gene	Name	Sequence (5'-3')	Length (bp)	Reference
Rotavirus A	NSP3	Rota A-F Rota A-R Rota A-P	ACCATCTACACATGACCCTC GGTCACATAACGCCCC FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-TAMRA	87	(Pang et al., 2004)
Swine influenza A virus	M	M-F M-R M-P	**AGATGAGT**CTTCTAACCGAGGTCG TGCAAAGACACTTTCCAGTCTCTG FAM-TCAGGCCCCCTCAAAGCCGA-**BHQ1**	101	F and P modified from (Loeffen et al., 2011) R: this study
	H1 (A(H1N1)pdm09)	H1pdm-F H1pdm-R H1pdm-P	AATGCCGAACTGTTGGTTCT CAATTTCCTTGGCATTGTTTT FAM-CTGGCTTCTTACCTTTT*(BHQ1)CATATAAGTTCTTC	118	(Slomka et al., 2010)
Porcine circovirus type 2	CAP	PCV2-F PCV2-R PCV2-P	GATGATCTACTGAGACTGTGTGA AGAGCTTCTACAGCTGGGACA **FAM**-TCAGACCCCGTTGGAATGGTACTCCTC-**BHQ1**	152	Modified from (Ladekjær-Mikkelsen et al., 2002)
Porcine circovirus type 3	CAP	PCV3-F PCV3-R PCV3-P	AGTGCTCCCCATTGAACG ACACAGCCGTTACTTCAC **FAM**-ACCCCATGGCTCAACACATATGACC-**BHQ1**	135	Modified from (Palinski et al., 2017)
Porcine parvovirus	NS1	PPV-F PPV-R PPV-P	ACCGCCAGATTCAGCA**R**TAC ACCTTATTCAAGGTTTGTTGTGGG FAM-CACCAAAGCAGGCTCTTATGTCGGTTTCTA-BHQ1	113	F and P from Dia[1] R: this study
Porcine reproductive and respiratory syndrome virus type 1 (EU)	ORF7	PRklm EU1-F PRklm EU1-R PRklm EU1-P	GCACCACCTCACCCRRAC CAGTTCCTGCRCCYTGAT FAM-CCTCTGYYTGCAATCGATCCAGAC-BHQ1	77	(Wernike et al., 2012)
	ORF6	PRklm EU2-F PRklm EU2-R PRklm EU2-P	CAGATGCAGAYTGTGTTGCCT TGGAGDCCTGCAGCACTTTC FAM-ATACATTCTGGCCCCTGCCCAYCACGT-BHQ1	78	(Wernike et al., 2012)
	ORF5	Nadir-F Nadir-R Nadir-P	TTYGGGTTCACHGTCGCAG GACCTTCGATARTTCGGGAG FAM-CAGAGCGCGAACGGAGAAKCGCG-BHQ1	108	(Stadejek et al., 2017a)
Porcine reproductive and respiratory syndrome virus type 2 (US)	ORF7	PRklm NA-F PRklm NA-R PRklm NA-P	ATRATGRGCTGGCATTC ACACGGTCGCCCTAATTG **FAM**-TGTGGTGAATGGCACTGATTGACA-**BHQ1**	114	Modified from (Kleiboeker et al., 2005)
Porcine Cytomegalovirus	DPOL	Pol-F Pol-R Pol-P	CTGCCGTGTCTCCCTCTAG ATTGTTGATAAAGTCACTCGTCTGC FAM-CCATCACCAGCATAGGGCGGGAC-**BHQ1**	81	Modified from (Fryer et al., 2004)

[1] Dia = The diagnostic veterinary laboratory at the National Veterinary Institute, Technical University of Denmark

Table 2 Primer and probe sequences for bacteria detection. Modifications of published sequences are in bold

Pathogen - Bacteria	Target gene	Name	Sequence (5'-3')	Length (bp)	Reference
B. pilosicoli	23S rRNA	B.pilo-F	GTAGTCGATGGGAAACAGGT	124	(Ståhl et al., 2011)
		B.pilo-R	TTACTCACCACAAGTCTCGG		
		B.pilo-P	FAM-TATTCGACGAGGATAACCATCACCT-BHQ1		
L. intracellularis	16S rRNA	Law-F	GCGCGCGTAGGTGGTTATAT	98	(Lindecrona et al., 2002)
		Law-R	GCCACCCTCTCCGATACTCA		
		Law-P	FAM-CACCGCTTAACGGTGGAACAGCCTT-TAMRA		
***E. coli* type F4**	*faeG*	E.coli F4-F	CACTGGCAATTGCTGCATCT	86	(Frydendahl et al., 2001)
		E.coli F4-R	ACCACCGATATCGACCGAAC		
		E.coli F4-P	FAM-TCACCAGTCATCCAGGCATGTGCC-TAMRA		
***E. coli* type F18**	*fedA*	E.coli F18-F	GGCGGTTGTGCTTCCTTGT	128	(Frydendahl et al., 2001)
		E.coli F18-R	CCGTTCACGGTTTTCAGAGC		
		E.coli F18-P	FAM-TAACTGCCCGCTCCAAGTTATATC AGCTGTT-TAMRA		
M. hyopneumoniae		Mhp-F	GGCAATTCCAAGAGTTATTCAGG	147	This study
		Mhp-R	TTCCGACAAGTTTTTCACCATTAG		
		Mhp-P	FAM-TGATGGACTAATTGATAAAGTTCTAAACCATCG-BHQ1		
A. pleuropneumoniae	*omlA*	AP-F	AGTGCTTACCGCATGTAGTGGC	92	(Angen et al., 2001)
		AP-R	TTGGTGCGGACATATCAACCTTA		
		AP-P	FAM-CGATGAACCCGATGAGCCGCC-TAMRA		
P. multocida	*kmt1*	PM-F	GACTACCGACAAGCCCAC	123	R from (Townsend et al., 1998) F and P: this study
		PM-R	ATCCGCTATTTACCCAGTGG		
		PM-P	FAM-GTGCGAATGAACCGATTGCCGCG-BHQ1		
***S. suis* type 2**	*gdh*	SS-F	CCAAAGCTTCATGACTGAATTGC	81	Modified from (Yang et al., 2010)
		SS-R	CGACCACCGACACCGATG		
		SS-P	FAM-ACACATCGGACCTTCACTTGACG**T**C-BHQ1		
B. bronchiseptica	*dnt*	BB-F	**G**GCGGTACTTGGGATAATAGA	136	F modified from (Stepniewska et al., 2010) R and P: This study
		BB-R	GAAGAGTCCGGGGATCTTG		
		BB-P	FAM-CGAGCATCCTGGCCGATGGGTTC-BHQ1		
M. hyorhinis	*p37*	Mhr-F	CAAGCTTCYGAAACACCAAATG	115	This study
		Mhr-R	CGCCAATAGCATTTGCTATATC		
		Mhr-P	FAM-CAGGAGTAGTCAAGCAAGAGGATG-BHQ1		

Table 3 Relative sensitivity of qPCR assays on the Rotor-Gene Q platform and on the diagnostic 48.48DA (BioMark platform)

	Assays									
Bacteria dilution	***B. pilosicoli***		***L. intracellularis***		***E. coli* F4**		***E. coli* F18**		***P. multocida***	
	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark
10	15.53	6.96	14.84	5.88	17.03	8.26	19.84	11.01	11.1	4.40
10^{-1}	18.80	10.41	18.03	8.99	20.26	10.90	23.07	14.74	15.63	6.90
10^{-2}	22.19	13.68	21.31	12.40	23.56	14.54	26.61	18.29	18.39	9.32
10^{-3}	25.78	16.66	24.65	16.30	27.07	17.99	30.05	22.29	22.35	13.21
10^{-4}	28.99	20.13	28.30	19.66	30.03	21.43	33.17	25.52	24.82	16.54
10^{-5}	32.50	24.75	31.68	23.23	33.78	24.57	35.91	neg	27.93	20.07
10^{-6}	neg	neg	neg	25.54	neg	neg	neg	neg	31.76	neg
10^{-7}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Effectivity	0.97	0.95	0.98	0.96	1.00	1.00	1.03	0.89	1.00	1.06

	Assays									
Bacteria dilution	***B. bronchiseptica***		***S. Suis* type 2**		***A. pleuropneumoniae***		***M. hyorhinis***		***M. hyopneumoniae***	
	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark
10	-	-	-	-	8.72	(3.68)*	13.27	5.99	22.52	12.10
10^{-1}	16.06	5.71	12.89	5.06	11.44	(3.83)*	16.31	8.63	25.30	15.40
10^{-2}	19.56	7.46	16.11	7.75	14.70	5.98	19.52	11.67	29.26	19.68
10^{-3}	22.94	10.91	19.85	10.82	18.00	9.14	22.75	15.88	32.86	22.98
10^{-4}	26.73	14.44	22.90	14.39	21.64	12.40	25.99	19.02	36.17	neg
10^{-5}	30.02	17.75	26.40	16.99	25.30	16.17	29.60	21.74	neg	neg
10^{-6}	33.56	20.72	29.93	20.47	28.49	19.26	33.10	24.73	neg	neg
10^{-7}	neg	25.18	32.99	24.66	32.06	22.38	neg	neg	neg	neg
10^{-8}	neg	neg	neg	(25.90)*	34.29	neg	neg	neg	neg	neg
10^{-9}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Effectivity	0.93	1.06	0.97	1.06	1.00	1.01	1.01	1.01	0.94	0.85

* Number in parenthesis is not included in the effectivity calculation

Table 3 continued Relative sensitivity of qPCR assays on the Rotor-Gene Q platform and on the diagnostic 48.48DA (BioMark platform)

	Assays											
Viral dilution	**swIAV (M)**		**swIAV (H1pdm)**		**Rotavirus A**		**PCV2**		**PCV3**		**PPV**	
	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark
10	-	-	-	-	22.19	14.50	14.01	5.18	17.33	7.27	18.7	9.61
10^{-1}	13.69	3.68	16.42	14.02	25.55	18.05	17.24	7.76	20.51	9.60	22.12	13.32
10^{-2}	17.25	5.18	19.95	17.41	28.73	20.64	20.59	11.09	24.50	13.32	25.92	17.21
10^{-3}	20.69	8.62	23.51	20.21	32.13	24.65	23.94	14.67	27.41	16.30	29.27	20.84
10^{-4}	24.15	12.67	27.13	24.03	(34.86)*	neg	27.48	17.60	30.98	19.83	33.51	24.17
10^{-5}	26.91	16.17	30.25	27.61	neg	neg	31.22	20.73	34.35	22.51	36.46	neg
10^{-6}	29.32	19.71	33.48	neg	neg	neg	34.03	24.40	36.60	26.32	neg	neg
10^{-7}	neg	23.51	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
10^{-8}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Effectivity	1.07	0.96	0.96	0.96	1.01	1.03	0.97	1.04	1.00	1.05	0.89	0.87

* Number in parenthesis is not included in the effectivity calculation

	Assays									
Viral dilution	**PRRSV type 1 (EU1)**		**PRRSV type 1 (EU2)**		**PRRSV type 1 (Nadir)**		**PRRSV type 2 (NA)**		**PCMV**	
	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark
10	19.17	13.54	-	10.17	14.69	10.86	-	16.48	12.15	3.50
10^{-1}	22.01	17.27	20.74	14.33	18.29	14.35	21.51	20.59	15.76	6.12
10^{-2}	25.41	19.91	23.57	17.49	21.58	17.38	24.44	24.47	18.71	9.43
10^{-3}	28.53	22.51	27.51	20.20	25.19	20.71	28.14	27.60	21.93	12.84
10^{-4}	32.09	26.31	30.79	23.63	28.75	25.35	31.79	31.19	25.66	15.80
10^{-5}	35.72	28.02	33.76	28.50	32.18	neg	34.08	neg	29.22	19.33
10^{-6}	neg	neg	37.29	neg	neg	neg	neg	neg	31.8	22.60
10^{-7}	neg	neg	neg	neg	neg	neg	neg	neg	33.97	neg
10^{-8}	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Effectivity	0.99	1.10	1.00	0.93	0.93	0.96	1.03	1.00	1.06	1.02

Table 4 Faecal sock samples analysed on the Rotor-Gene Q and BioMark platforms. - = no Cq, PCC = Pearson correlation coefficient. Differences are marked in red

Sample	*B. pilosicoli*		*L. intracellularis*		*E. coli* F4		*E. coli* F18	
	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark
17-18303-1	-	-	26.47	19.81	-	-	-	-
17-18304-1	-	-	-	-	29.24	21.33	26.90	23.44
17-18305-1	-	-	-	-	-	-	26.07	21.62
17-18306-1	25.68	23.41	26.19	18.31	-	-	-	-
17-18307-1	-	-	-	-	30.29	22.29	25.58	22.79
17-18308-1	29.13	31.17	25.02	15.28	-	-	-	-
17-18309-1	-	-	-	-	-	-	25.51	19.36
17-18310-1	-	-	28.73	20.69	-	-	-	-
17-18311-1	-	-	29.7	21.66	30.48	23.77	24.90	19.60
17-18312-1	-	-	-	26.56	-	-	30.88	28.68
17-18313-1	-	-	-	-	-	-	23.49	18.48
17-18315-1	-	-	22.83	14.23	29.79	22.03	26.66	23.10
17-18316-1	28.6	-	24.99	17.93	28.53	22.09	25.86	30.95
17-18317-1	-	-	20.74	11.93	-	-	-	-
17-18318-1	24.76	21.53	-	-	-	-	-	-
17-18319-1	27.90	29.46	29.28	20.88	33.05	25.48	29.17	25.55
17-18320-1	25.72	19.24	25.94	17.67	-	-	37.29	34.11
17-18321-1	25.63	-	25.46	18.45	-	-	36.74	-
17-19768-1	-	-	30.71	21.51	-	-	28.76	25.53
17-19769-1	-	-	28.98	21.48	-	-	-	-
17-19770-1	-	-	-	-	28.84	21.05	-	-
17-19771-1	-	-	25.00	15.41	-	-	27.01	22.80
17-19772-1	22.99	16.39	23.94	14.67	-	-	21.20	14.71
17-19773-1	24.06	18.94	24.55	15.47	-	-	32.13	29.75
17-19774-1	23.98	17.66	22.94	14.17	-	-	-	-
17-19775-1	24.76	18.04	20.06	11.45	-	-	-	-
17-19776-1	26.59	21.57	24.27	15.34	-	-	-	-
17-19777-1	30.15	25.08	19.88	10.81	-	-	-	-
17-19778-1	25.77	22.17	29.86	20.26	-	-	30.19	26.19
17-19779-1	32.07	26.94	35.34	25.55	-	-	30.07	24.67
17-19780-1	28.25	22.58	20.86	11.66	-	-	-	-
17-19781-1	30.96	24.43	29.02	19.76	-	-	-	-
17-19782-1	25.90	21.17	22.53	13.47	-	-	-	-
17-19783-1	30.86	26.06	22.89	13.41	-	-	-	-
17-19783-2	24.54	19.11	33.32	23.66	-	-	30.99	24.69
17-19783-3	29.38	32.91	-	-	-	-	-	-
In total	21/36	19/36	27/36	28/36	7/36	7/36	19/36	18/36
PCC	0.771		0.976		0.918		0.845	
p-value	0.0001		0.00001		0.004		0.00001	

Table 5 Lung tissue samples analysed on the Rotor-Gene Q and BioMark platforms. mas = massive (Cq value < 25), mod = moderate (Cq value 25-30), low (Cq value 30-33), - = negative, grey = no analysis. Differences are marked in red

		A. pleuropneumoniae		*M. hyorhinis*		*M. hyopneumoniae*		*P. multocida*		PRRSV type 2	
Sample	Material	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark	Rotor-Gene	BioMark
89-1	Lung	-	-	-	-	+	19.01	mas	10.90		
89-2	Lung	-	-	-	-	+	23.85	mas	5.89		
89-3	Lung	-	-	-	-	+	16.80	mas	15.82		
443-1	Lung	-	-	mas	8.65	-	-	mas	15.06		
710-1	Lung	-	-	mas	11.65	-	-	-	-		
710-2	Lung	-	-	low	25.79	-	-	-	-		
710-3	Lung	-	-	mas	9.60	-	-	-	-		
1275-1	Lung	mas	10.26	-	-	-	-	low	24.16		
1275-2	Lung	mod	19.01	-	-	-	-	-	-		
1908-1	Lung	mod	18.72	mas	7.65	-	-	mas	12.56		
1908-2	Lung	mas	6.31	mas	13.81	-	-	mas	10.45		
1908-3	Lung	mas	15.18	mas	15.15	-	-	mas	20.81		
1991-1	Lung	-	-	mas	10.25	-	-	mas	11.06		
1991-2	Lung	-	-	mas	7.96	-	-	mod	21.80		
1991-3	Lung	-	-	mas	11.84	-	-	mas	-		
2511-1	Lung	mas	14.87	mas	16.45	-	-	low	-		
2512-1	Lung	mod	17.85	-	-	-	-	-	-		
2512-2	Lung	mas	12.01	-	-	-	-	-	-		
2512-3	Lung	mas	4.64	-	-	-	-	-	-		
2919-1	Lung	mas	16.38	low	-	-	-	-	-		
2919-2	Lung	low	21.75	mas	16.22	-	-	-	-		
2985-1	Lung	-	-	-	-	+	17.93	mas	15.25		
2985-2	Lung	-	-	-	-	+	14.29	mas	13.71		
2985-3	Lung	-	-	-	-	+	19.83	mas	11.33		
3148-1	Lung	-	-	-	-	+	19.61	mod	19.94		
3148-2	Lung	-	-	-	-	+	14.01	-	30.46		
3148-3	Lung	-	-	-	-	+	21.36	-	24.97		
3368-1	Lung	low	22.37	low	25.89	+	12.83	low	-		
3368-2	Lung	mas	16.75	-	-	+	18.56	mod	18.27		
3368-3	Lung	mas	9.30	-	27.56	+	18.03	mas	12.44		
455-5	Lung									26.2	26.07
455-6	Lung									31.2	-
3386-1	Lung		-		-	+	16.61	mas	16.57		
3953-2	Lung		-		19.34	-	-	low	-		
3953-3	Lung		-		16.79	-	-	mas	11.90		
4506-1	Lung		24.19		-	+	13.32	-	26.62		
4506-2	Lung		20.70		-	-	-	mas	18.46		
4506-3	Lung		4.69		17.81	+	16.02	mas	10.18		

Table 6 Lung tissue samples analysed on the BioMark platform. - = negative, grey = no analysis

		Diagnostic 48.48 DA (BioMark platform)				
Sample	Material	PCMV	PCV2	*S. Suis* type 2	*B. bronchiseptica*	PCV3
89-1	Lung	-	18.52	25.83	-	19.34
89-2	Lung	22.14	-	20.22	-	18.95
89-3	Lung	-	-	-	-	-
443-1	Lung	11.52	-	21.01	12.05	-
710-1	Lung	17.01	-	-	-	-
710-2	Lung	10.33	-	27.76	-	-
710-3	Lung	9.44	-	24.09	-	-
1275-1	Lung	21.74	-	27.83	-	14.78
1275-2	Lung	-	27.33	-	-	21.06
1908-1	Lung	17.22	21.69	21.85	-	-
1908-2	Lung	12.16	22.46	20.21	-	-
1908-3	Lung	18.08	26.52	26.70	-	-
1991-1	Lung	11.21	-	18.85	-	-
1991-2	Lung	12.99	-	27.15	-	-
1991-3	Lung	13.67	-	21.06	-	-
2511-1	Lung	21.55	6.69	-	-	-
2512-1	Lung	19.16	-	-	27.00	-
2512-2	Lung	7.91	-	23.78	21.33	-
2512-3	Lung	18.30	-	27.24	26.04	-
2919-1	Lung	13.66	-	-	-	-
2919-2	Lung	15.38	-	19.08	-	-
2985-1	Lung	-	-	-	-	-
2985-2	Lung	24.31	-	25.93	-	29.55
2985-3	Lung	-	-	-	-	-
3148-1	Lung	23.21	-	27.77	-	-
3148-2	Lung	23.90	-	-	-	-
3148-3	Lung	22.44	13.03	26.77	-	17.75
3368-1	Lung	14.36	-	21.83	-	17.93
3368-2	Lung	18.00	-	24.18	-	-
3368-3	Lung	16.13	-	19.23	-	-
455-5	Lung					
455-6	Lung					
3386-1	Lung	26.77	21.17	-	-	19.92
3953-2	Lung	8.18	-	25.10	15.63	-
3953-3	Lung	11.21	-	24.34	15.76	-
4506-1	Lung	18.55	-	15.82	-	-
4506-2	Lung	14.10	-	21.06	26.73	-
4506-3	Lung	23.38	-	14.90	23.40	15.73

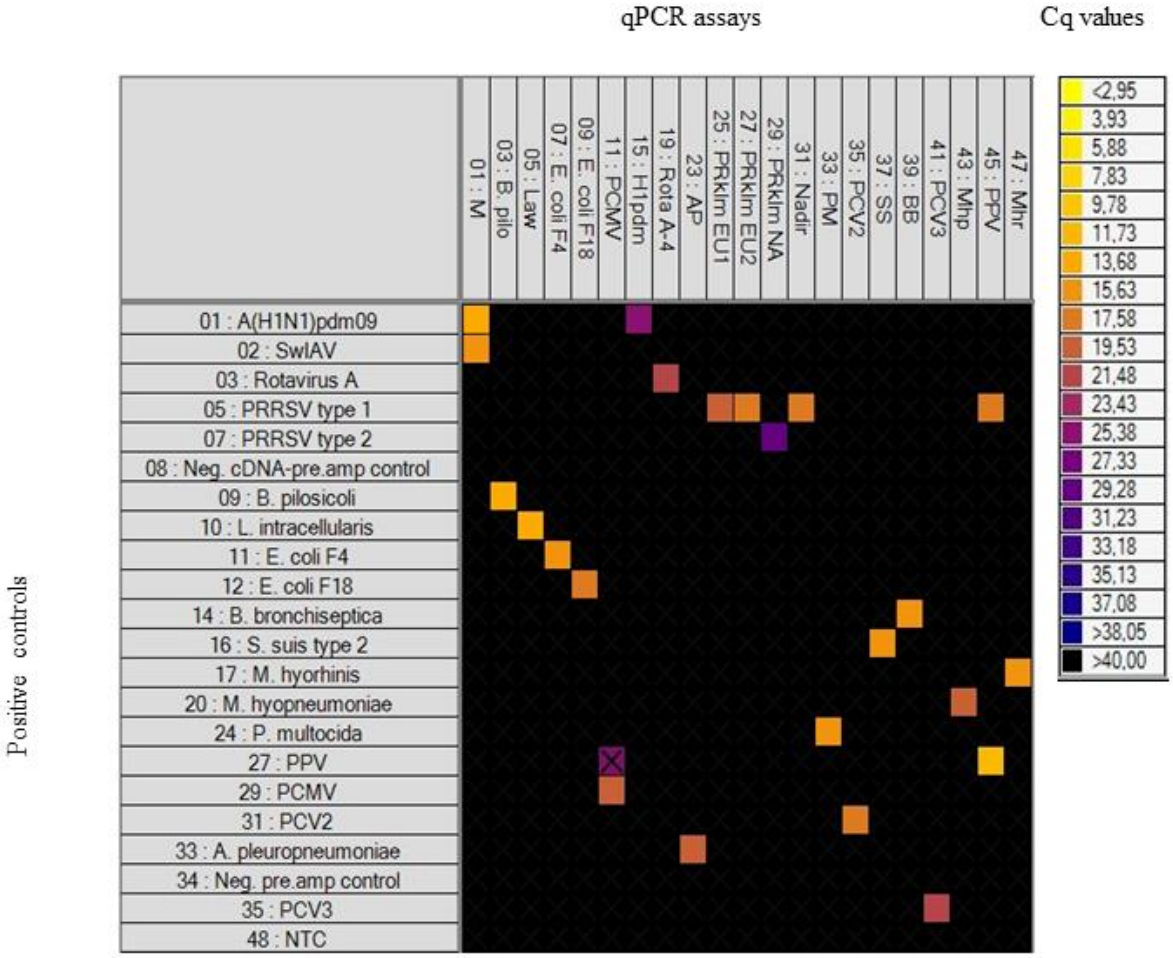

Figure 1 Heat map showing the specificity of the qPCR assays included on the diagnostic 48.48DA by testing known positive samples (controls). At the top: The qPCR assays (Table 1 and 2). To the left: The positive samples (controls), a Non-Template Control (NTC), a negative cDNA/pre-amplification control and a negative pre-amplification control. Each square corresponds to a single real-time PCR reaction. Cq values for each reaction are indicated by colour; the corresponding colour scale is presented in the legend on the right. A black square is considered as a negative result. The black cross is shown if the amplification curve deviates too much from an ideal amplification curve.

3.3 Manuscript III

Objective health monitoring in nursery and finisher pigs by extended laboratory diagnostic testing

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(In preparation)

Abstract

Infectious diseases are of great economic importance in pig production, causing both clinical and subclinical disease, and influences welfare, productivity, and antibiotic use. The causes of these diseases are often multifactorial and laboratory diagnostics are not routinely performed. The aim of the current study was to explore the benefits of monthly health monitoring in nursery and finisher herds and to examine correlations between laboratory results and observed clinical signs, including coughing and diarrhoea. Three monthly samplings were conducted in three different age groups in six nursery and four finisher herds. For each herds, two pens were randomly selected in each age group and evaluated for coughing and diarrhoea events. Furthermore, faecal sock and oral fluid samples were collected in the selected pens and analysed for 17 respiratory and enteric viral and bacterial pathogens using the high-throughput real-time PCR BioMark platform (Fluidigm). A total of 174 pens were sampled in which eight coughing events and 77 diarrhoeic events were observed. The overall findings showed that swine influenza A virus, porcine circovirus 2, porcine cytomegalovirus, *Brachyspira pilosicoli*, *Lawsonia intracellularis*, *Escherichia coli* fimbria type F4 and F18 were found to be prevalent in several of the herds. Significant correlations between coughing events and the presence of swine influenza A virus, porcine cytomegalovirus ($Cq \leq 20$) or a combination of these were found. Furthermore, a significant correlation between diarrhoeic events and the presence of *L. intracellularis* ($Cq \leq 24$) or *B. pilosicoli* ($Cq \leq 26$) was found. The use of high-throughput real-time PCR analysis for continuous monitoring of pathogens and thereby dynamics of disease in a pig herd, provided the veterinarian and farmer with an objective knowledge on the distribution of pathogens in the herd. In addition, the use of a high-throughput method in combination with information about clinical signs, productivity, health status and antibiotic consumption, presents a new and innovative way of diagnosing and monitoring pig herds and even to a lower cost than the traditional method.

Keywords

Diagnostics, monitoring, high-throughput real-time PCR, coughing index, diarrhoea index, respiratory viruses, respiratory bacteria, enteric viruses, enteric bacteria.

Introduction

Infectious diseases such as respiratory and intestinal diseases are of major importance in pig production due to clinical and subclinical diseases, which can result in reduced productivity, impaired animal welfare, increased mortality and increased consumption of antibiotics. The cause of these diseases are often multifactorial and the prevalence and combination of pathogens can fluctuate over time due to e.g. changes in management, environment, season or stage of production (Hansen et al., 2010; Heo et al., 2013; Hernandez-Garcia et al., 2017; Opriessnig et al., 2011; Stärk, 2000).

Respiratory diseases are one of the major problems in modern pig production worldwide and are often referred to as Porcine Respiratory Disease Complex (PRDC), which is a polymicrobial infection caused by a combination of various primary and secondary respiratory viral and bacterial pathogens. Environmental conditions, management factors, population size and factors such as age and genetic also play roles in the outcome of PRDC (Opriessnig et al., 2011; Thacker, 2001). Agents associated with PRDC are e.g. porcine circovirus type 2 (PCV2), porcine cytomegalovirus (PCMV), porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza A virus (swIAV), *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Pasteurella multocida* and *Streptococcus suis*. PRDC is most commonly observed in growing and finishing pigs, with mortality rates ranging from 2-10% and morbidity rates ranging from 10-40%. The clinical picture of the complex is characterized by coughing, fever, dyspnoea, decreased feed intake and even fatal pneumonia (Brockmeier et al., 2002; Harms et al., 2002; Kim et al., 2003; Opriessnig et al., 2011; Thacker, 2001).

Pathogens involved in respiratory disease in pigs vary among countries, herds and production sites, making general treatment and control regimes for PRDC difficult to develop (Gutiérrez-Martín et al., 2000; Harms et al., 2002). Due to the polymicrobial nature of the disease, a range of different diagnostic samples and techniques may be employed in the investigation of a single case, including nucleic acid, antigen and antibody detection. For this, oral fluid samples are ideal for diagnosing PRDC due to the residency of the pathogens in the respiratory tract (Hernandez-Garcia et al., 2017). The use of oral fluid as a sampling method is a relatively new diagnostic method used for detection of pathogens. This sampling method has the advantage that it is practical, minimizes stress on the animals, offers the possibility of testing a large number of individuals in an aggregate sample and is economically beneficial compared to blood sample collection (Gibert et al., 2017;

Hernandez-Garcia et al., 2017; Prickett and Zimmerman, 2010). On the other hand, analysing oral fluid samples can be challenging due to the risk of contamination from faeces, nasal secretions or from the environment. Furthermore, isolating RNA from these biological samples can be difficult as it is easily degraded. Therefore, pre-processing and storage conditions such as time and temperature can be critical in the analysis of oral fluid samples (Gibert et al., 2017; Hernandez-Garcia et al., 2017).

Intestinal diseases are another critical factor in modern pig production. The multifactorial disease "post-weaning diarrhoea" is associated with dehydration, reduced feed intake and thereby reduced growth and increased mortality (Heo et al., 2013). Intestinal diseases in pigs can be caused by a wide range of viral and bacterial pathogens. The most frequently detected pathogens associated with intestinal disease in nursery pigs are *Lawsonia intracellularis*, *Brachyspira pilosicoli*, *Escherichia coli* fimbria type F4 and F18, whereas for finisher pigs especially *L. intracellularis* and *B. pilosicoli* are involved in enteric disease (Jacobson et al., 2003; Pedersen et al., 2014). The viruses; rotavirus A and PCV2 can also cause enteric disease. Rotavirus A is a known diarrhoea causing agent in pigs and has been associated with acute gastroenteritis, usually seen in young animals (Bohl et al., 1978; Saif and Fernandez, 1996). PCV2 has not been proven to be the primary cause of diarrhoea in pigs, however, systemic PCV2 may indirectly contribute to enteric diseases due to its immunosuppressive effect (Jensen et al., 2006; Johansen et al., 2013; Segalés, 2012). Coronaviruses such as transmissible gastroenteritis virus, porcine deltacoronavirus and porcine epidemic diarrhoea virus can also induce enteric diseases, but Denmark and many other European countries are free of these viruses (Pensaert and Martelli, 2016).

The use of laboratory diagnostics in Danish pig production is limited and is often carried out only once a year, which is a requirement before batch medication can be used according to Danish legislation. The results of the diagnostic tests of these random cross-sectional samples are often of limited value and therefore, prophylactic and therapeutic interventions are often initiated based solely on clinical signs. The major reason for the limited use of laboratory diagnostics is that the traditional methods are expensive and resource demanding. To overcome these limitations, we have developed a high-throughput real-time PCR (qPCR) detection system using the BioMark platform (Fluidigm, South San Francisco, USA) (Goecke et al., 2018), which is capable of detecting significant porcine viruses and bacteria in the same setup.

The aim of the present study was to test if ongoing monthly diagnostic monitoring of pathogens on herd level could be used as a supportive tool in veterinary consultancy, combined with clinical

observations and production data, to create a more objective basis for intervention. Furthermore, the study aimed to investigate the prevalence of the different pathogens and their relation to clinical disease with a special focus on pathogens involved in PRDC and enteric diseases in pigs after weaning.

Materials and methods

Study design and herd inclusion criteria

The study was carried out as a repeated cross-sectional study in ten pig herds located on Zealand, Denmark. The target populations of each herd consisted of three different age groups in the nursery and finisher pigs. For each herd, sampling and clinical registrations were conducted monthly over a three months period from September to November 2017. Six nursery (1N, 2N, 3N, 4N, 5N and 6N) and four finisher (1F, 2F, 3F and 6F) herds were included in the study, of which herds 2, 3, 4, 5 and 6 were specific pathogen free (SPF) herds and herd 1 was a conventional herd. All herds were free of PRRSV at the beginning of the study. General information on the herds and their vaccination strategies was obtained from questionnaires filed out by the herd owners (Table 1).

Sampling strategy and clinical observation

At each sampling, three different age groups in each herd were sampled (Figure 1). In the nursery herds, pigs were sampled approximately two, five, and seven weeks after weaning. In the finisher herds, pigs were sampled approximately two, six, and ten weeks after arrival. Samplings were always conducted at the same location/herd if a farm consisted of multiple finisher herds, occasionally resulting in a deviation from the sampling age with two weeks. To reduce the spread of disease, the youngest pigs were always sampled first. Two pens from each age group were randomly included in the study, representing this age group. By lottery, one pen was first chosen. To minimize environmental factors and to maximize the distance between the study pens, the other pen was chosen by turning to the pen just opposite of the aisle and then move a defined number of pens downwards, calculated by dividing the number of pens in a row by two (Figure 1). In herd 5N, four nursery pens were included per age group due to the small numbers of pigs housed per pen. Hospital pens were excluded from the study. Due to the cross-sectional study design, the same pens were not target to be sampled at the next sampling month, although this could happen by coincidence.

At each sampling, the coughing index, which is a method to quantify coughing in groups of pigs, was calculated (Nathues et al., 2012). The pigs in the study pens were counted and forced to move if sleeping. Coughs were then counted for three minutes. If the same pig was coughing more than once within a ten second period, this coughing episode was noted, however, only counted once in the total number of coughs. Based on the coughing index, a coughing event was defined to determine if the pen was a coughing or a non-coughing pen. If the coughing index was ≥ 0.2, the pen was defined as having a coughing event (Tolstrup et al., 2017). Similarly, the incidence of diarrhoea was evaluated at each sampling by using a descriptive classification scale described by Pedersen and Toft, 2011 (Pedersen and Toft, 2011). Here, a diarrhoeic event was calculated and used to classify whether a pen had diarrhoea or not (Tolstrup et al., 2018).

Sampling of oral fluid and faecal sock samples

In total, 172 oral fluid samples were collected from nursery and finisher pigs through the application of an oral fluid sampling kit (Dianova, Kgs. Lyngby, Denmark). Oral fluid sampling was performed pen-wise by tying a cotton rope to the gate of the pen 20-30 cm above the floor (height adjusted to the size of the pigs). For approximately 30 min, the pigs were allowed to chew on the rope and thereby deposit oral fluid in the rope. After 30 min, the rope was collected and placed in a plastic bag, squeezed and oral fluid extracted in a 10 mL centrifuge tube. To minimize cross-contamination between samples, disposable gloves and new scalpels were used for each pen.

In addition, 174 faecal sock samples were collected from nursery and finisher pigs by means of a sock sampling kit (Dianova). One sock sampling kit was used per pen. Faecal sock samples were acquired by treading through the faecal contaminated part of the pen wearing the sampling socks as previously described (Pedersen et al., 2015).

Oral fluid and faecal sock samples were marked with specific identification numbers with information on pen, herd, age group, sample type, and date. All samples were stored in a polystyrene box containing freezer packs before delivery to the National Veterinary Institute, Technical University of Denmark. After delivery, all samples were kept under cooled conditions in a refrigerator at approximately 5 degrees for a maximum of 48 hours until preparation. Oral fluid samples were dispensed and stored at -80°C for RNA and DNA extraction. For each of the faecal sock samples, a 10% faeces dilution was prepared in phosphate buffered saline (PBS) and stored at -20°C for RNA and DNA extraction.

Nucleic acid extraction

Oral fluid samples were extracted using the extraction robot QIAcube HT (QIAGEN, Hilden, Germany) and Cador pathogen 96 QIAcube HT kit (QIAGEN). The Cador pathogen 96 QIAcube HT protocol (QIAGEN) was applied with the following modifications; the input volume was increased from 200 to 400 µL and the volume of lysis buffer VXL was increased from 100 to 200 µL. Before extraction, 1000 µL of each oral fluid sample was centrifuged for 5 min at 9000 x g at room temperature (15–25°C) and 400 µL of the supernatant was used for extraction together with positive and negative (nuclease-free water, Amresco) controls. The nucleic acid extractions were stored at -80°C until further analysis.

The 10% faeces dilution samples were extracted using the QIAsymphony SP system (QIAGEN) extraction robot and QIAsymphony DSP virus/pathogen mini kit (QIAGEN) following the manufacturer's instructions. For this, the protocol Complex200_V5_DSP was used with an elution volume of 110 µL. Prior to the nucleic acid extraction, one 5 mm steel bead was added to each sample following which the samples were homogenized in a Tissuelyser II (QIAGEN) for 20 sec at 15 Hz. The homogenate was then centrifuged for 90 sec at 10,000 rpm and 350 µL of the supernatant was used for nucleic acid extraction together with positive and negative (nuclease-free water, Amresco) controls. The nucleic acid extractions were stored at -80°C until further analysis.

Pathogen detection by high-throughput real-time PCR

Extracted oral fluid and faecal sock samples were both reverse transcribed/pre-amplified and pre-amplified as previously described (Goecke et al., 2018). For high-throughput qPCR amplification, the BioMark 48.48 dynamic array (48.48DA) system (Fluidigm, South San Francisco, USA) was used, which combines 48 pre-amplified samples with 48 assays for 2,304 individual and simultaneous qPCR reactions. The qPCR assays and procedure used for the high-throughput qPCR analysis in the present study have previously been described and validated in the study by Goecke et al., 2018 (Goecke et al., 2018). qPCR assays were performed in duplicates and samples were performed in single reactions. Three positive controls, containing positive controls for the included assays, two non-template controls (nuclease-free water, Amresco), a non-template cDNA/pre-amplification and a non-template pre-amplification control (nuclease-free water, Amresco) were included on each 48.48DA to control for non-specific amplification and sample contamination.

Statistical analysis

The correlation between the presence of a pathogen and clinical signs in a pen was investigated using chi-square test or Fishers exact probability test, if cell frequencies were less than five. For the respiratory pathogens, the Fishers exact probability test (two-tailed) was used with the following parameters; presence vs. absence of pathogens and coughing event vs. no coughing event. For the intestinal pathogens, the chi-square test (with Yates correction) was used with the following parameters; presence vs. absence of pathogens and diarrhoeic event vs. no diarrhoeic event. For comparison of the mean quantification cycle (Cq) values between September, October and November 2017 the one-way analysis of variance (ANOVA) test and the t-test were used. The analyses were performed using Graph Pad Prism version 7.0.

Results

In total, 174 pens in ten herds were included in the study. In addition to the clinical registrations of coughing and diarrhoea, 172 oral fluid and 174 faecal sock samples were collected. The clinical registrations recorded eight coughing events (4.7% of the pens) during the three months of sampling, and the events were only observed in the nursery pigs. Furthermore, 77 of the collected pens were defined as pens with diarrhoeic events (44.3%) of which 53.2% of the nurseries and 46.8% of the finishers were affected. Registrations of mortality and medical treatments on pen or batch level were not carried out consistently throughout all the investigated herds, and therefore, these data could not be included in the study. In all herds, the diagnostic results were consistent with the SPF status of the herds. PRRSV type 1 and 2 were not detected in any of the herds.

For swIAV, two different assays were included in the high-throughput qPCR analysis, one general swIAV assay detecting all known subtypes of swIAV and an assay specific for the human pandemic H1 strain (A(H1N1)pdm09). Thus, a positive result on swIAV are reported as either "swIAV" if the general swIAV assay was positive and "H1 (A(H1N1)pdm09)" if both assays were positive for a given sample.

Individual herd analysis

<u>Herd 1: nursery (1N) and finisher (1F) pigs (Supplementary table 1A and B)</u>

In herd 1, three age groups were sampled in the nurseries, whereas only two age groups were sampled in the finishers due to continuous flow and no clear separation of age groups. In the early nursery period, coughing episodes were often observed, but a coughing event was only recorded in

one pen (marked with green in Supplementary table 1A). In the affected pen, swIAV, PCMV and *A. pleuropneumoniae* were detected. swIAV was detected in all age group in the nurseries, while it was only found sporadically in the finishers. *A. pleuropneumoniae* was found in all pens in October and November 2017 in the finishers, while it was only found in single pens in the other samplings. PCMV and *S. suis* type 2 were found to circulate in both nurseries and finishers in all pens. Furthermore, *M. hyorhinis* was detected in almost all pens. In general, piglets generally seemed small at weaning in this herd.

Seventeen diarrhoeic events (marked with red in Supplementary table 1A and 1B), distributed between the nurseries and finishers, although clinical diarrhoea was only observed in few pens after weaning. In general, *E. coli* F4 and *E. coli* F18 were detected in the beginning of the nursery period in all three months, while *L. intracellularis* was detected at the end of the nursery period. Rotavirus A was also present in several of the nursery pens. However, no clear association was observed between the presence of pathogen and diarrhoeic events. In the finisher pens, clinical diarrhoea was observed in almost all pens although intestinal pathogens were only detected in few of them.

Herd 2: nursery pigs (2N) (Supplementary table 1C)

It was not possible to collect oral fluid samples from the youngest pigs in November due to their lack of interests in chewing in the rope. Three coughing events (marked with green in Supplementary table 1C) were registered in this herd, all in the early nursery period in September and October. In these pens, swIAV and PCMV were detected with low Cq values. *S. suis* type 2 was circulating in all pens, while PCMV, PCV2, PCV3, *M. hyorhinis* and *A. pleuropneumoniae* were detected in many of the pens. Furthermore, four diarrhoeic events (marked with red in Supplementary table 1C) were detected in herd 2 and *L. intracellularis* was found in all four affected pens. Rotavirus A was found in nearly all pens, whereas *E. coli* F4 and *E. coli* 18 were found to be most prevalent in the beginning of the nursery period. *L. intracellularis* was found in the mid and late nursery period with lowest Cq values in November. In this herd, newly weaned pigs were fed liquid feed and extra water in troughs to optimize weaning, which may contribute to the low levels of intestinal pathogens and clinical diarrhoea observed in general.

Herd 3: nursery (3N) and finisher (3F) (Supplementary table 1D and 1E)

Coughing events were detected in two pens in the nurseries (marked with green in Supplementary table 1D). PCMV was detected in nearly all pens, but the virus was found with the lowest Cq values

in the affected pens. swIAV, which was found in one of the affected pens and was detected with low Cq values in the beginning of the nursery period in September and October, whereas in November it was detected in the middle to late nursery period. In contrast, swIAV was only detected sporadically in the finishers. PCV3, *M. hyorhinis* and *S. suis* type 2 were also detected in many of the pens. Four diarrhoeic events (marked with red in Supplementary table 1D) were registered in the nursery pens and eight diarrhoeic events (marked with red in Supplementary table 1E) were registered in the finishers. No clear pattern was observed for the findings of *B. pilosicoli*, *L. intracellularis*, *E. coli* F4 and *E. coli* 18, which were all detected sporadically in all age groups. Furthermore, no clear pattern between the pens affected by diarrhoea and the findings of pathogens was observed.

Herd 4: nursery (4N) and finisher (4F) pigs (Supplementary table 1F and 1G)

Clinically, no coughing events were observed in this herd during the three sampling months. This is in line with the finding that swIAV did not seem to cause problems in this herd. Between the samplings in October and November, the veterinarians experienced clinical signs of wasting and uneven weight distribution 15-25 days after arrival to the nursery. Multiple factors could be the reason for this. PCV2 was found in all age groups and with low Cq values in the finishers, and therefore, this virus could potentially have had an impact on productivity and secondary infections. Furthermore, *M. hyorhrinis*, *B. bronchiseptica* and PCV3 were found sporadically in the nurseries and finishers. Fifteen diarrhoeic events were observed (marked with red in Supplementary table 1F and 1G). *E. coli* F4 and *E. coli* F18 seemed to be a problem in the early nursery period. *B. pilosicoli*, sometimes in combination with *L. intracellularis*, dominated in the mid to late nursery period. Infections with *B. pilosicoli* and *L. intracellularis* seemed to extend to the finishing period. In November, all pens in the nurseries were positive for diarrhoeic events, which coincided with detection of *E. coli* F4 and *E. coli* F18 20 days after weaning. In addition, findings of *B. pilosicoli* and *L. intracellularis* 34 and 48 days after weaning could also explain the symptoms observed. In the finishers, detection of *B. pilosicoli* and *L. intracellularis*, both separately and in combination, generally correlated with diarrhoeic events. Rotavirus A was primarily detected in September and October in the nurseries.

Herd 5: nursery pigs (5N) (Supplementary table 1H)

One coughing event was detected in this herd (marked with green in Supplementary table 1H) and the affected pen was positive for swIAV and PCMV. Furthermore, coughing was noticed with high frequency in the youngest age group in all three months, even though it was not correlated to coughing events. In general, swIAV was detected with low Cq values at 12-15 days after weaning and with higher Cq values in the late nursery period in September and October. PCVM, *S. suis* type 2 and *M. hyorhrinis* were detected in all pens, while PCV2 was detected in almost all pens. Several pens were also positive for PCV3 and *B. bronchiseptica*. Diarrhoea was rarely observed and only one diarrhoeic event was observed (marked with red in Supplementary table 1H). In the affected pen, *B. pilosicoli* was detected with a low Cq value. *L. intracellularis* and rotavirus A were also present, but with higher Cq values. Rotavirus A was found in all most all pens, while *L. intracellularis* only was found in the late nursery period in all three samplings. *E. coli* F4 and *E. coli* F18 were found mainly in the early to mid nursery period.

Herd 6: nursery (6N) and finisher (6F) pigs (Supplementary table 1I and 1J)

One coughing event was noticed in this herd in the mid nursery period in November (marked with green in Supplementary table 1I). In the affected pen, PCMV and *A. pleuropneumoniae* was detected and this pen was the only pen in the nurseries in which *A. pleuropneumoniae* was found. However, *A. pleuropneumoniae* was widely distributed in the finishers. PCMV was detected in many of the pens, but with the lowest Cq values in the nurseries. PCV2 and *S. suis* type 2 were detected in all pens except for one, and PCV2 was present with low Cq values in several of the pens with the majority in the finishers. swIAV was detected in all age groups in the nurseries and was also detected in the beginning of the finisher period. One of the sample collected in the nurseries in September, was positive for H1 (A(H1N1)pdm09). PCV3 was present in all age groups. Diarrhoea seemed to be a problem in this herd, since 28 diarrhoeic events (marked with red in Supplementary table 1I and 1J) distributed between the nurseries and finishers were observed. *B. pilosicoli* and *L. intracellularis* were mainly detected in the mid to late nursery period, while *B. pilosicoli* was found in every pen in the finishers and here *L. intracellularis* was only found in beginning of the period. *E. coli* F18 was detected in the nursery pigs 15-17 days after weaning in the three months, while *E. coli* F4 was only present in November.

Pathogen findings and clinical signs

This section describes the presence and dynamics of pathogens across the herds and, when relevant, the correlation between pathogens and coughing events or diarrhoeic events was calculated.

Swine influenza A virus

swIAV was detected in all herds with an overall prevalence of 34.9%. The infection patterns varied between the herds and age groups. For most of the herds, swIAV was present shortly after weaning, and only sporadically later in the nursery period. In the finishers, only a few pens were found positive and mainly in the beginning of the period. Six out of eight pens, in which coughing events were registered, were found to be positive for swIAV and a significant correlation between coughing events and swIAV detection were found ($p = 0.02$). Furthermore, to investigate whether the level of swIAV correlated to the coughing events different cut-off Cq values were tested (Table 2). No significant correlation between coughing events and the level of swIAV was found with cut-off values of Cq 16 ($p = 0.08$) and 18 ($p = 0.10$), while with a cut-off value of Cq 20 a significant correlation was observed ($p = 0.03$). The Cq values for all the positive swIAV findings are plotted in Figure 2.

Porcine cytomegalovirus

PCMV was detected in almost all pens in all herds and had an overall prevalence of 92.4%. In general, the detection of PCMV was highly consistent with the lowest Cq values in the early to mid nursery period and with higher Cq values in the samples collected from six to eight weeks after weaning and until slaughtering (Figure 3). PCMV was detected in the eight pens in which coughing events were registered with Cq values between 8.0 and 16.3. Furthermore, there was a significant relationship between the level of PCMV and coughing events with cut-off Cq values of 16 ($p = 0.0001$), 18 ($p = 0.002$) and 20 ($p = 0.02$), while no significant correlation was observed with a cut-off value of 22 ($p = 0.21$) (Table 2). The Cq values for all the positive findings of PCMV are plotted in Figure 4.

In general, PCMV was present with lower Cq values in the pens with swIAV. Furthermore, PCMV with Cq value ≤ 20 was also detected in the six "coughing event" pens, which were positive for swIAV, and a significant correlation between these two viruses and coughing event was found ($p = 0.004$).

Porcine circovirus type 2

PCV2 was detected in all herds, but with different patterns. The virus was detected in the oral fluid samples in 59.9% and in 40.2% of the faecal sock samples. PCV2 was detected in four of the "coughing event" pens with Cq values ≥ 23, and no significant correlation between detection of PCV2 and coughing event was observed ($p = 0.72$). Since it has been showed that PCV2 can be related to intestinal disease the association with diarrhoeic events was also investigated (Segalés et al., 2005). PCV2 was detected in 41.6% of the pens, where diarrhoeic events were observed with Cq values between 10.2 and 27.5. No significant correlation was observed between the presence of PCV2 and diarrhoeic events ($p = 0.87$), even when using different cut-off values (Table 3).

Porcine circovirus type 3

PCV3 was detected in all herds with the majority of Cq values above 20. The virus was detected sporadically in most of the herds, but more frequently in herd 3. In the oral fluid samples, the overall prevalence of PCV3 was 53.5%. For the faecal sock samples, it was 16.1%. PCV3 was detected in five of the "coughing event" pens, where four of the pens had Cq values above 25, while the last one had a Cq value of 19, and no significant correlation was observed between the presence of PCV3 and coughing events ($p = 0.73$). PCV3 was also detected in 16.1% of the faecal sock samples and was present in 16.9% of the pens, where diarrhoeic events were registered, with Cq values above 23.

Actinobacillus pleuropneumoniae

A. pleuropneumoniae was detected in five of the ten herds with an overall prevalence of 17.4%. *A. pleuropneumoniae* was found in three of the eight pens, where coughing events were observed, with Cq values above 24. There was no significant correlation between *A. pleuropneumoniae* positive pens and coughing events ($p = 0.15$).

Mycoplasma hyohrinis

M. hyohrinis was frequently detected in herds 1 to 5 with the majority of Cq values above 20, while it was not detected in herd 6. *M. hyohrinis* was detected with an overall prevalence of 59.3% and with a different detection patterns for the different herds. In herds 1N, 1F, 2N, and 5N almost all pens were positive, while a more sporadic distribution was found in herds 3N, 3F, 4N, and 4F. *M.*

hyorhinis was present in seven of the "coughing event" pens with Cq values above 20 and no significant correlation between *M. hyorhinis* detection and coughing event was found (p = 0.08). In Figure 5, Cq values from the positive samples were plotted and a decrease in Cq values was observed during the three months, with the highest mean Cq in September (25) and the lowest mean Cq in November (23). An one-way ANOVA test confirmed that the mean Cq values for the three months were not equal. Furthermore, a t-test analysis showed that there was a significant difference between the mean Cq values for the three months: September and October (p = 0.03), October and November (p = 0.01) and September and November (p = 0.00001).

Streptococcus suis type 2

S. suis type 2 was detected in all herds with an overall prevalence of 98.8%. *S. suis* type 2 was found in all eight pens in which coughing events were observed with Cq values above 21. No significant correlation was found between *S. suis* type 2 detection and coughing event (p = 0.91).

Mycoplasma hyopneumoniae, Bordetella bronchiseptica and *Pasteurella multocida*

M. hyopneumoniae was only found in two pens (1.2%) and both in herd 6F in the sampling from October. *B. bronchiseptica* and *P. multocida* were detected sporadically with the prevalence of 25.0% and 15.1%, respectively. However, none of these bacteria were detected in pens with coughing events.

Brachyspira pilosicoli

B. pilosicoli was found with an overall prevalence of 39.7% and was generally found from mid nursery to late finishers period. *B. pilosicoli* was detected in all herds except for herd 1, and furthermore was present in 37 (48.1%) of the tested pens, in which diarrhoeic event was also observed. A significant correlation between diarrhoeic events and the presence of *B. pilosicoli* was observed when using a cut-off Cq value ≤ 26 (p = 0.03) (Table 3).

Lawsonia intracellularis

L. intracellularis was detected with an overall prevalence of 40.8% and was found mainly in the mid to late nursery and finisher period. *L. intracellularis* was detected in 37 (48.1%) of the pens, where diarrhoeic event was observed. For *L. intracellularis*, a significant correlation between the

presence of the bacterium and diarrhoeic events was found when using a cut-off Cq value ≤ 24 (p = 0.01) (Table 3).

Escherichia coli F4 and F18

E. coli F4 and *E. coli* F18 were detected in 19.0% and 28.7% of the pens, respectively, and mainly in the beginning of the nursery period, although a sporadic detection was also observed in the finishers. In general, *E. coli* F18 was detected more frequently than *E. coli* F4 in the herds except for herds 2N and 4N. *E. coli* F4 was present in 12 (15.6%) of the tested pens, in which diarrhoeic event was also observed, while for *E. coli* F18 the number of pens was 16 (20.8%). For both *E. coli* F4 and *E. coli* F18, no significant correlation was found to pens with diarrhoeic events (p = 0.43 and p = 0.06, respectively), even when using different cut-off Cq values (Table 3).

Rotavirus A

Rotavirus A was detected in all herds with an overall prevalence of 54.0%. In general, rotavirus A was most frequently detected in the beginning of the nursery period, in which also the lowest Cq values were found. In the youngest age group of the nurseries, only one pen (herd 4N) was negative. In the finishers, Rotavirus A was only detected sporadically. The virus was detected in 29 (37.7%) of the pens, where a diarrhoeic event was observed. No significant correlation between the presence of rotavirus A and diarrhoeic events was found when using a cut-off Cq value ≤ 20 (p = 0.3), however, a significant correlation was observed when using Cq values above ≥ 22 (p = 0.009) (Table 3).

Discussion

In the present study, three monthly samplings were conducted in six nursery and four finisher herds to investigate the value of continuous screening for selected respiratory or enteric viral and bacterial pathogens in different age groups. Oral fluid and faecal sock samples were collected and analysed using the high-throughput diagnostic system described elsewhere (Goecke et al., 2018). The use of a high-throughput qPCR platform, in which multiple samples can be analysed in different assays simultaneously, provides new possibilities for conducting extended diagnostics at a limited cost. The aim of the present study was to investigate the pathogen patterns in ten herds over time and compare the findings with the observed clinical signs of coughing and diarrhoea.

The vision of the diagnostic system tested in the present study, was that the herd consultant includes the results of the diagnostic screenings as a tool in the herd health management by benchmarking the monthly data on the presence and dynamics of pathogens with figures on productivity, feed consumption/feed plans, clinical symptoms and antibiotic consumption. By that the herd consultant will be able to identify the underlining course of impaired production figures or health. Furthermore, in case of acute outbreak of clinical disease or sudden change in performance, samples are often submitted for diagnostic examinations; however, the results of these tests are often difficult to interpret because most of the potential pathogens are often circulating in herds without clinical impact. If screening of the herd had been performed during the months prior to the acute outbreak, the "outbreak" results can be compared to these herd-specific historical data and by that identify if a given pathogen has been introduced or has changed dynamics. In all of the ten herds included in the present study, the screening data identified unexpected pathogen patterns and by that identified potential targets for preventive measures to increase the health and/or productivity.

In herd 1, swIAV was circulating in the herd in all age groups, which was probably due to the continuous flow of pigs in the nursery rooms and/or the continuous production of finishers without washing and disinfection of pens between batches. Only new gilts and gilts prior to farrowing were vaccinated against swIAV (Respiporc FLU3, IDT), however, vaccination of piglets should be considered based on the screening results. Furthermore, *E. coli* F4 and *E. coli* F18 were often detected in this herd without correlation to disease. However, enteritis can be present in a herd without causing clinical signs (Weber et al., 2017), and therefore, vaccination targeting *E. coli* for nursery pigs may be beneficial.

In herd 2, swIAV was present in all age group with low Cq values but the H1 (A(H1N1)pdm09) strain was not detected at any sampling point. The herd vaccinated against A(H1N1)pdm09 (FluSure pandemic, Zoetis), but this vaccine does not cross protect against enzootic Danish swIAVs, and therefore, vaccination against other swIAV subtype(s) could be effective in this herd.

In herd 3, there was a long-term history of swIAV infection and, therefore, an intensive vaccination protocol was carried out, in which piglets, new gilts, gilts and sows were vaccinated (Respiporc FLU3, IDT). Despite the vaccination, swIAV was still found to be present in the early to mid nursery period and in the finishers 14-16 days after arrival. The continued circulation of swIAV could be due to infection with a heterologous subtype(s) not included in the vaccine or a short-term

effect of the piglet vaccination. Therefore, a subtyping of the circulating swIAV subtype(s) should be carried out to determine if the applied vaccine is specific enough. Furthermore, it could be considered to vaccinate sows 3-4 times a year to ensure high levels of immunity in the sow herd.

In herd 4, the enteric pathogens *E. coli* F4, *E. coli* F18, *B. pilosicoli* and *L. intracellularis* were detected in several of the pens. In addition, PCV2 was present in all age groups, and especially in the finisher. Furthermore, the average weight gain in the finishers were below the Danish national average. Thus, these data implies that PCV2 has a negative impact in this herd, and therefore, vaccination against PCV2 should be initiated.

In herd 5, a comprehensive vaccination protocol was applied against swIAV (Respiporc FLU3, IDT), including vaccination of piglets, new gilts before introduction, gilts and sows. Despite this vaccination regime, swIAV was still detected, which could indicate that the effect of vaccination was suboptimal. To address this, subtyping or sequencing of the circulating swIAV strains should be performed to secure that the vaccine elicited cross protection. Furthermore, the herd had a poor productivity (ADWG at 385 g/day) and the nursery mortality was 4%. PCV2 vaccination was performed at weaning, but still moderate levels of PCV2 were found in the youngest nursery pigs and therefore it cannot be excluded that PCV2 has an influence on the poor productivity. Thus, it may be beneficial in this herd to perform PCV2 vaccination at an earlier time point.

In herd 6, new gilts were vaccinated against A(H1N1)pdm09 (FluSure pandemic, Zoetis) and swIAV (Respiporc FLU3, IDT) before introduction. H1 (A(H1N1)pdm09) was detected in one pen, while other swIAV subtype(s) was found to be present sporadically in several of the pens, mainly in the nursery herd. Subtyping of the circulating swIAV subtype(s) should be performed to examine if the vaccination could be more effective. PCV2 was widely distributed in both the nurseries and finishers and in levels which could cause systemic disease and lead to decreased productivity (Segalés, 2012). Vaccination against PCV2 was not applied in this herd, but based on the findings in the present study, vaccination before weaning could be considered to control PCV2.

M. hyopneumoniae was detected in two pens in the finisher herd despite vaccination. In the finishers, a mortality rate of 4.5% was found based on the data from the productivity report. The mortality could be due to concurrent circulation of several pathogens, but more investigations are needed to confirm this. A high stocking density was often noticed during the samplings, which could contribute to the on-going circulation of these pathogens. High occurrence of diarrhoea seemed to be a clinical issue in both nurseries and finishers with a clear pattern. *E coli* F4 and/or *E coli* F18 were found in the beginning of the nursery period followed by *L. intracellularis* and *B.*

pilosicoli, which persisted until slaughter. Common practise in this herd was to use water medication pen wise, however, an extension to batch medication may be more effective. Furthermore, focus should be on optimizing diets, hygiene, stocking density in the finishers. *E. coli* F4 and *E coli* F18 vaccination could be considered.

The coughing events observed in the present study seemed to correlate with the presence of swIAV ($p = 0.02$) or PCMV ($Cq \leq 20$) ($p = 0.02$) or a combination of these ($p = 0.004$). The correlation between coughing and isolation of swIAV has been shown in another study which used the same cut-off for coughing events (Tolstrup et al., 2017). However, the clinical effects of PCMV are not clear and a cut-off Cq value was also needed in the present study in order to obtain a significant correlation to coughing events. To our knowledge, co-infection with swIAV and PCMV has not previously been described, and further studies are needed to support this finding. However, the correlation between coughing events and the presence of pathogen was based on a few coughing events, which makes it difficult to make clear conclusions.

swIAV was detected in all the tested herds, and all herds, except for herd 4, vaccinated against swIAV using different vaccination strategies. Among the herds, different infection patterns were observed, however, the most typical pattern was high levels of swIAV just after weaning, which could indicate poor immunity. The lack of immunity may be explained by short-term duration of maternal immunity or reduced effect of vaccination. The reduced effect of vaccination might be due to the high evolution rate of swIAV, which is able to undergo changes doing antigenic drift or shift. As a consequence, the swIAV strain is no longer recognized by the vaccine as it will differ from the swIAV strains included in the vaccine (Carrat and Flahault, 2007; Zambon, 1999).

PCV2 was found with a high prevalence in herds 4F, 5N, 6N, and 6F. Although no correlation to neither coughing nor diarrhoeic events was evident, PCV2 might still act sub-clinically. Furthermore, reasonable good management practise was seen in all herds which, combined with high health status, could minimize the impact of PCV2. The role of PCV2 in PRDC is still up for discussion. Studies suggest that PCV2 lung lesions do not exist without systemic infection and may only participate in PRDC sub-clinically (Raith et al., 2015; Ticó et al., 2013). This could explain the missing correlation between PCV2 and clinical respiratory signs in the present study. Furthermore, a study suggested that PCV2 is more often found in connection with PRRSV than with swIAV and *M. hyopneumoniae* (Harms et al., 2002), however, PRRSV was not detected in this study.

PCV3 is a novel discovered virus and only a limited number of studies are available. The current study is to our knowledge the first to demonstrate the wide distribution of PCV3 in Danish pig herds. PCV3 was found in all age groups in both nurseries and finishers and was primarily detected with relatively high Cq values. Furthermore, it did not correlate to events of respiratory disease.

There is sparse documentation on the predictive value of detecting bacteria in oral fluid samples and therefore oral fluid is recommended only as a screening tool and the detection of these bacteria should be confirmed by traditional diagnostic tests (i.e. culturing from lungs of dead pigs). *M. hyorhinis, S. suis* type 2, *B. bronchiseptica*, and *P. multocida* are all considered to be secondary invaders in relation to PRDC and commensals present in both healthy and diseased pigs. In this study, *S. suis* type 2 and *M. hyorhinis* were indeed detected with relatively high Cq values in all pens (except herd 6 for *M. hyorhinis*) and no clinical signs could be correlated to the detection of these two pathogens. *B. bronchiseptica* and *P. multocida* were found more sporadically. These bacteria were also detected with high Cq values and they were not found in relation to clinical disease. Interestingly, the mean Cq values of *M. hyorhinis* differed significantly between all three sampling months, with the highest Cq mean recorded in September and the lowest Cq mean in November. This could indicate a seasonal variation in the infection pressure of *M. hyorhinis* although the clinical effect is unknown. A benefit of continuous health monitoring could potentially be the detection of such seasonal variances for more of the analysed pathogens if evaluated for an extended period.

A. pleuropneumoniae and *M. hyopneumoniae* act as primary pathogens, providing optimal conditions for secondary pathogens. *A. pleuropneumoniae* was only found in the oral fluids in herds already declared positive and was only detected with high Cq values, which could indicate that carrier animals harboured *A. pleuropneumoniae* in the tonsils. Another explanation could be that the sensitivity of detecting *A. pleuropneumoniae* by PCR in oral fluid compared to lungs is considered low (Gottschalk, 2015). However, the bacterium was not detected in correlation to clinical signs in this study. *M. hyopneumoniae* was rarely detected in this study. A study found limited sensitivity of detecting *M. hyopneumoniae* in oral fluid, which could lead to an underestimation in positive pens (Hernandez-Garcia et al., 2017).

For some of the herds, a clear pattern of enteric pathogens was observed, in which *E. coli* F4 and/or *E. coli* F18 were present in the early nursery period, *L. intracellularis* in the mid nursery to early finishing period and *B. pilosicoli* in the late nursery to late finisher period. This distribution of

these bacteria is comparable to others findings (Jacobson et al., 2003; Stege et al., 2000; van Beers-Schreurs et al., 1992). For other of the herds, no specific pattern was found. Diarrhoeic events observed in the present study seem to be associated with different bacteria in different age groups and with a similar pattern. However, no significant correlation between diarrhoeic events and the presence of *E. coli* F4 and *E. coli* F18 was found. On the other hand, a significant correlation was observed for *L. intracellularis* (Cq ≤ 24) and *B. pilosicoli* (Cq ≤ 26). Furthermore, rotavirus A was detected in all herds and especially in the nurseries. This virus is known to be endemic in pig herds and has been associated with acute gastroenteritis in young animals (Saif and Fernandez, 1996). In the present study, rotavirus A was found in several of the pens affected by diarrhoeic events, however, a significant correlation between diarrhoeic event and the presence of the virus was only observed when including Cq values above 22 ($p = 0.009$). Due to the fact that Rotavirus A only seems to pose a problem when including the high Cq values, this virus was probably not the cause of diarrhoea in the affected pens.

Diarrhoeic events were also observed in pens where no intestinal pathogens were found indicating that the course was non-infectious or caused by pathogens, which were not included in the high-throughput analysis. Another study found that approximately 50% of the investigated pigs suffering from diarrhoea were negative for pathogenic intestinal bacteria when using qPCR (Weber et al., 2015).

In general, the findings in the present study were based on Cq values, which gives an indication of how positive the sample is. However, quantitative measurements of pathogen load, including e.g. copies/g faeces, will provide a more informative result, since it may be able to differentiate a colonisation pathogen from a disease causing pathogen (Yang and Rothman, 2004). For several of the intestinal bacteria, cut-off values have been proposed (Pedersen et al., 2014), and these values can be used to examine if a given bacteria is the main reason for the observed clinical disease or not.

In conclusion, the use of high-throughput qPCR analysis for monthly monitoring of pathogens and thereby dynamics of pathogens in a pig herd, provides the veterinarian and farmer with an objective knowledge on the distribution of pathogens in the herd. In addition, the use of a high-throughput method in combination with information about clinical signs, productivity, health status and antibiotic consumption, presents a new and innovative way of diagnosing and monitoring pig herds and even to a lower cost than the traditional method. Furthermore, this continuous monitoring of

pathogens in Danish pigs provide a tool for optimized preventive measures and by that could contribute to a reduction of antibiotic consumption.

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Table 1 Information and vaccination strategies for each of the herds. N: nursery, F: finisher

	1N	1F	2N	3N	3F	4N	4F	5N	6N	6F
SPF status	Unknown	Unknown	Blue + AP12	Blue	Blue	Blue + M. hyo	Blue + M. hyo	Blue + M. hyo	Blue + M. hyo+ AP12	Blue + M. hyo+ AP12
swIAV status	Not diagnosed	Not diagnosed	Positive + A(H1N1)pdm09	Positive	Positive	Positive	Positive	Positive	Positive + A(H1N1)pdm09	Positive + A(H1N1)pdm09
Sows per year	400	400	770	560	560	-	2,500	2,500	735	730
No. of pen units	1,800	600	3,000	1,500 + 1,400	?	2,020	2,880	2,500	?	1,700
No. of produced 30 kg pigs per year	13,500	-	33,000	19,000 (sell 5500 per year)	-	20,000	-	80,000	23,500	-
No. of produced finishing pigs per year	-	4,000	-	-	13,500	-	12,300	-	-	6,000
Type of farm	Full-line	Full-line	Full-line	Full-line	Full-line	Nursery and finishers	Nursery and finishers	Sows and nursery	Full-line	Full-line
No. of sites	1 nursery site (total 1)	1 finisher site (total 1)	2 nursery sites (total 7)	2 nursery sites (total 3)	2 finisher sites (total 3)	1 nursery site (total 3)	3 finisher sites (total 3)	2 nursery sites (total 3)	1 nursery site (total 1)	1 finisher site (total 1)
Production	Weekly	Weekly	Weekly	Every 14th day	Every 14th day	Every 14th day	Every 14th day	Weekly	Weekly	Weekly
Vaccination status										
Erysipelothrix rhusiopathiae		+								
***Erysipelothrix rhusiopathiae* + parvovirus**		+		+				+		+
Glässers disease (*H. parasuis*)		+								
swIAV		+		+				+		+
A(H1N1)pdm09			+							+
M. hyopneumoniae			+				+			+
PCV2				+				+		
E. coli* + *C. perfringens		+	+	+				+		+
***E. coli* F4/F18**			+	+						

Table 2 p-values (Fishers exact probability test) calculated for different cut-off Cq values for respiratory viruses

Coughing event	**+/-**	**Cq ≤ 16**	**Cq ≤ 18**	**Cq ≤ 20**	**Cq ≤ 22**
p-value (swIAV)	0.02	0.08	0.10	0.03	na
p-value (PCMV)	0.53	0.0001	0.002	0.02	0.21

na: no analysis

Table 3 p-values (chi-square test) calculated for different cut-off Cq values for the intestinal pathogens

Diarrhoeic event	+/-	Cq ≤ 16	Cq ≤ 18	Cq ≤ 20	Cq ≤ 22	Cq ≤ 24	Cq ≤ 26	Cq ≤ 27
p-value (PCV2)	0.87	0.30	0.48	0.08	0.14	na	na	na
p-value (*B. pilosicoli*)	0.06	na	0.005	0.001	0.007	0.004	0.03	0.06
p-value (*L. intracellularis*)	0.12	na	0.006	0.008	0.03	0.01	0.91	na
p-value (*E. coli* F4)	0.43	na	na	0.82	0.43	na	na	na
p-value (*E. coli* F18)	0.06	na	na	na	0.82	0.58	na	na
p-value (Rotavirus A)	0.0002	na	na	0.3	0.009	0.007	na	na

na: no analysis

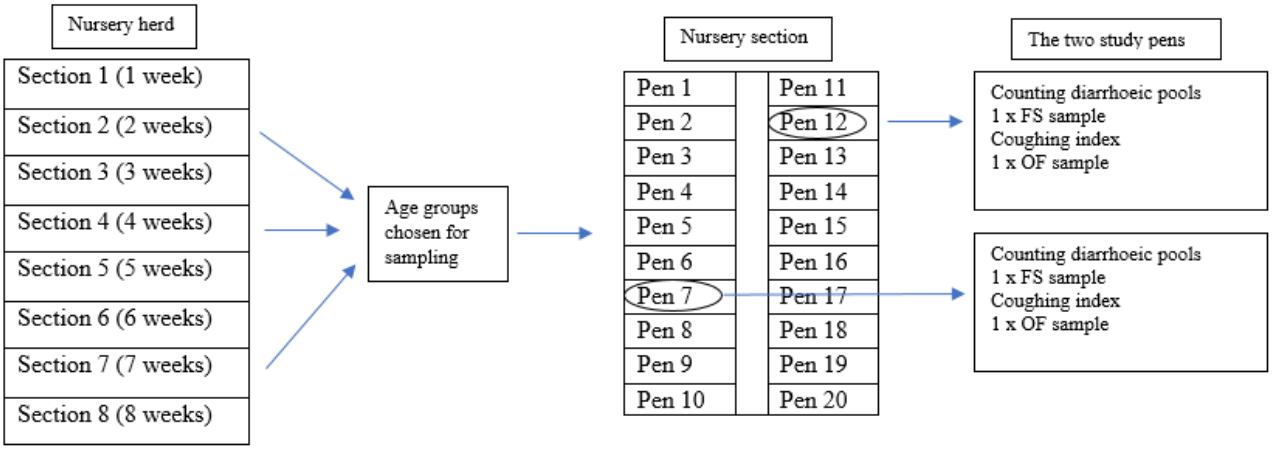

Figure 1 Schematic illustration of how age groups and pens were selected. Here, a nursery herd is used as an example. FS: faecal sock sample, OF: oral fluid sample.

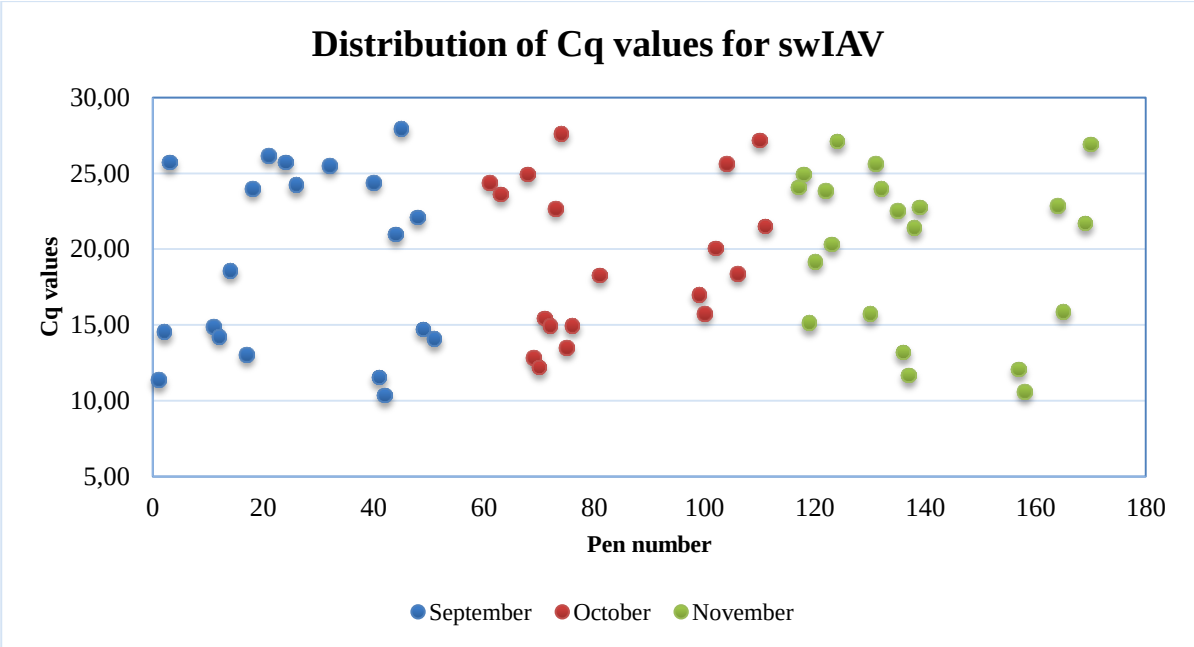

Figure 2 Cq values for each positive swIAV sample are plotted for each of the three sampling months (negative pens are not shown).

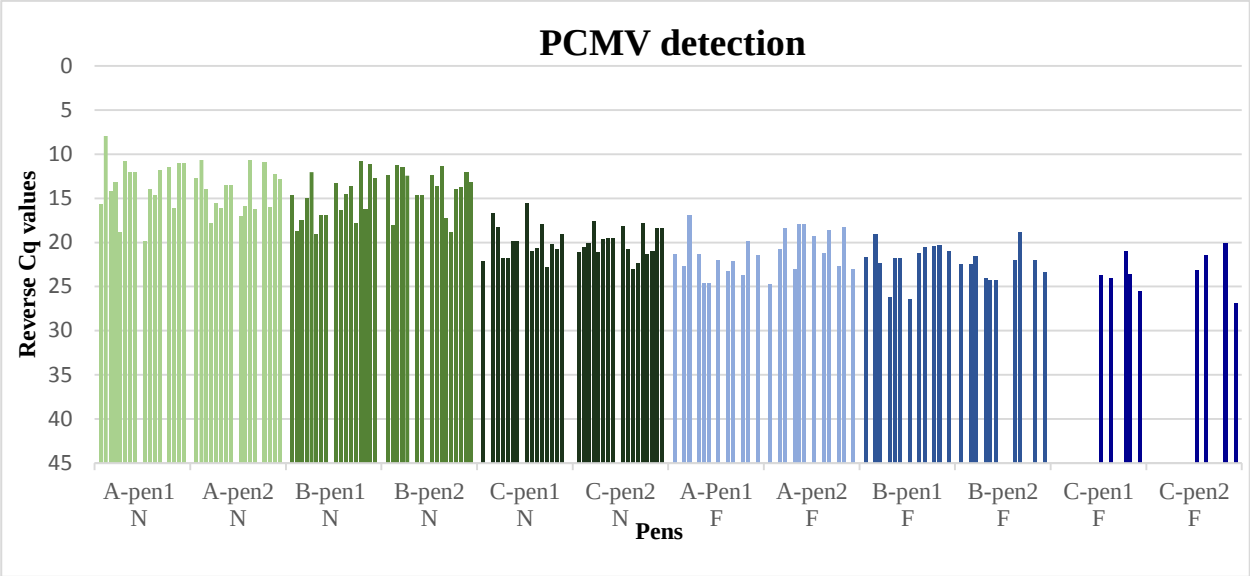

Figure 3 The reverse Cq values for the positive PCMV samples from all the pens are plotted. The samples are listed from youngest to oldest. A, B and C refer to the youngest, mid and oldest pigs, respectively, in both nurseries (N) and finishers (F). The y-axis shows the reverse Cq values.

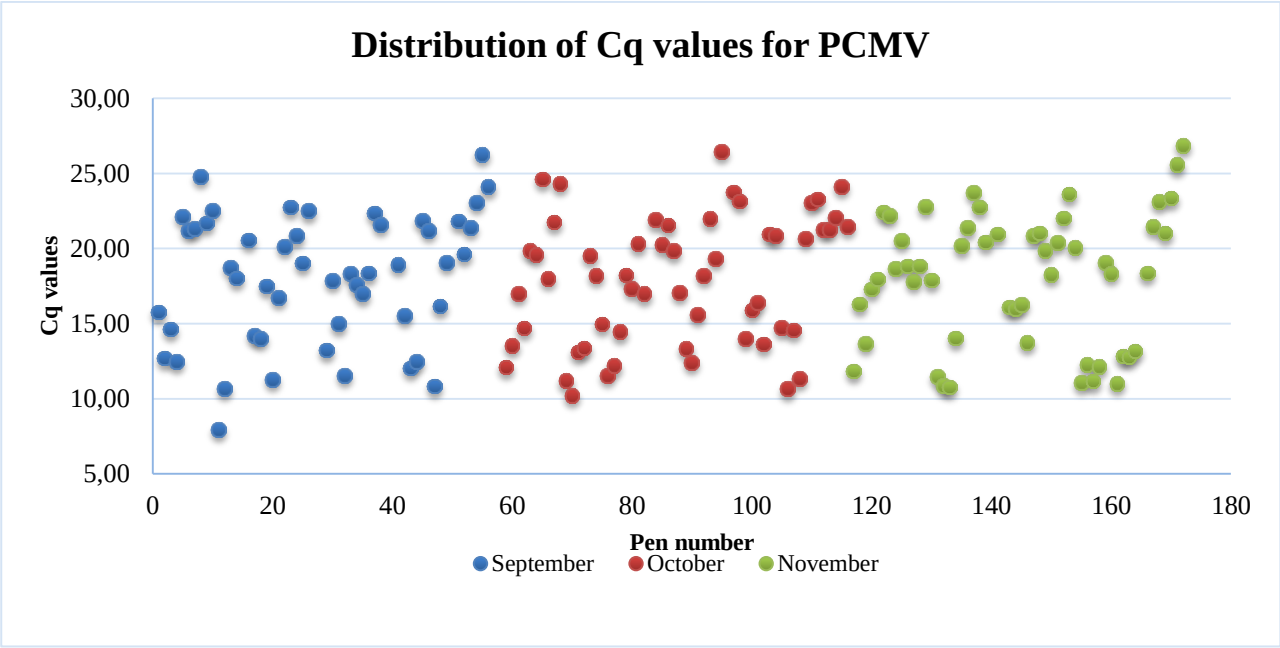

Figure 4 Cq values for each positive PCMV sample are plotted for each of the three sampling months (negative pens are not shown).

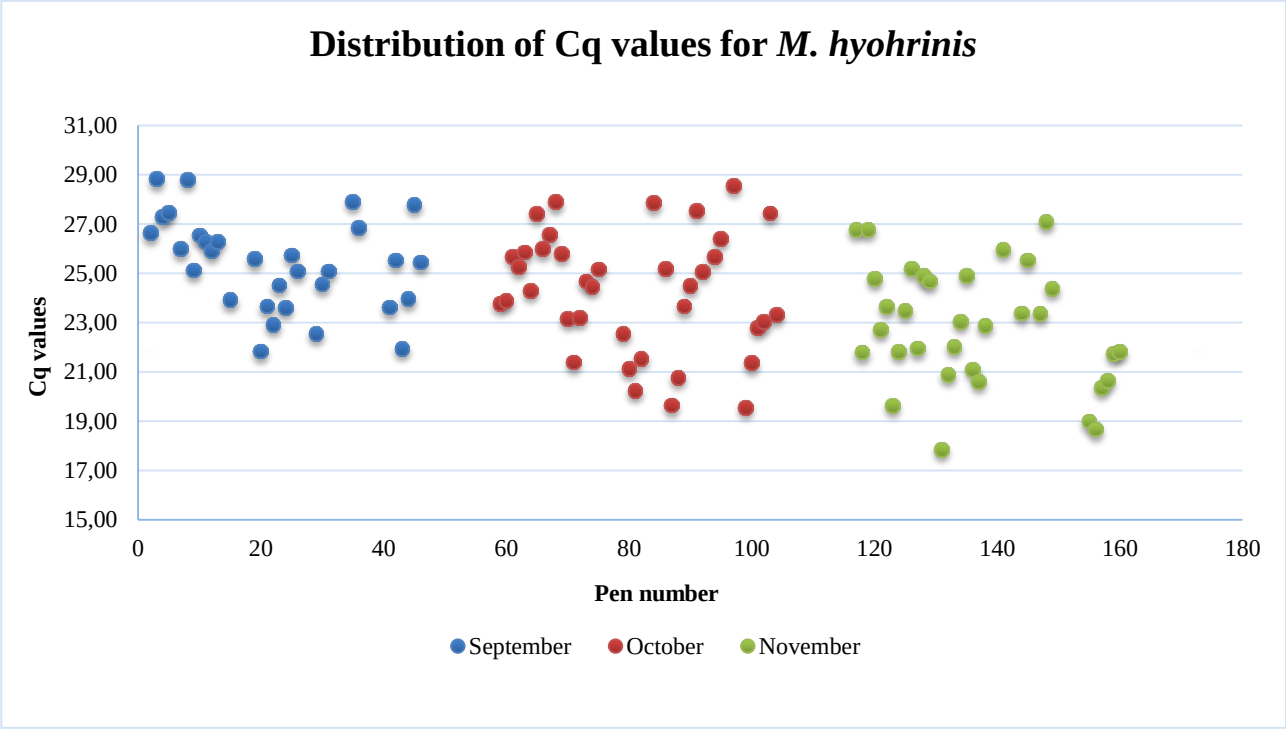

Figure 5 Cq values for M. hyohrinis positive sample are plotted for each of the three months (negative pens are not shown).

Supplementary tables

A) 1N – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		10		25		45		19		26		47		10		24		45	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF	11.4	14.5	25.7						24.4		23.6		24.1	24.9	15.1	19.2		23.9
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	15.8	12.7	14.6	12.5	22.1	21.2	12.1	13.5	17.0	14.7	19.9	19.6	11.8	16.3	13.7	17.3	18.0	22.4
A. pleuropneumoniae	OF					24.4									26.8				
PCV2	OF							27.5		21.6						27.0		23.3	
S. suis type 2	OF	22.5	22.8	26.2	26.6	21.9	22.2	24.4	22.6	22.9	23.6	24.3	23.22	24.6	21.1	23.1	24.4	17.4	19.6
B. bronchiseptica	OF					25.9				27.7		25.5							
PCV3	OF			26.6				25.1	24.8					17.3	18.7	27.9			30.0
PPV	OF																		
M. hyopneumoniae	OF																		
M. hyorhinis	OF		26.6	28.8	27.3	27.5		23.8	23.9	25.7	25.3	25.9	24.3	26.8	21.8	26.8	24.8	22.7	23.7
P. multocida	OF									29.9	29.8							27.9	
B. pilosicoli	FS																		
L. intracellularis	FS					19.2	21.1					25.0	22.8					17.0	
E. coli F4	FS		23.5				27.4		17.6					20.6	19.9				
E. coli F18	FS	18.2	21.2					20.8	15.6					16.4	15.5				
PCV3	FS													23.5	26.7				
PCV2	FS									26.9								27.5	
Rotavirus A	FS	27.9	27.4			30.5		21.2	17.4		22.9			17.7	17.1	24.6	21.5	29.7	

B) 1F – oral fluid (OF) and faecal sock (FS) samples													
Sampling date		September				October				November			
Days after insertion		? (45-80 kg)		? (80-100 kg)		? (45-60 kg)		? (50-85 kg)		? (65 kg)		? (75-80 kg)	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF								24.9	20.3	27.1		
H1 (A(H1N1)pdm09)	OF												
PRRSV type 1	OF												
PRRSV type 2	OF												
PCMV	OF	21.3	24.8	21.7	22.5	24.6	18.0	21.7	24.3	22.2	18.7	20.5	18.8
A. pleuropneumoniae	OF				23.9	24.3	22.9	24.8	22.2	22.9	22.9	23.1	22.8
PCV2	OF		24.7		25.0								25.7
S. suis type 2	OF	22.5	22.9	22.1	21.3	24.6	22.7	22.0	21.5	19.8	20.2	20.6	21.1
B. bronchiseptica	OF												
PCV3	OF			27.1	26.3				27.5	27.3	30.2		31.4
PPV	OF												
M. hyopneumoniae	OF												
M. hyorhinis	OF	26.0	28.8	25.1	26.5	27.4	26.0	26.6	27.9	19.6	21.8	23.5	25.2
P. multocida	OF					30.4			29.2				
B. pilosicoli	FS												
L. intracellularis	FS						21.8	25.0					
E. coli F4	FS												
E. coli F18	FS					24.6	24.7	24.8					
PCV3	FS												
PCV2	FS												
Rotavirus A	FS		26.4			24.5	31.4		27.1				

C) 2N – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		9		37		65		10		24		38		2		29-36		48	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF	14.9	14.2		18.6			12.8	12.2	15.4	14.9	22.7	27.6				15.7	25.6	24.0
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	8.0	10.7	18.7	18.0		20.6	11.2	10.2	13.1	13.4	19.5	18.2			17.8	18.8	22.8	17.9
A. pleuropneumoniae	OF							24.0	24.3	24.4	25.2	24.7					23.4		
PCV2	OF	24.6		18.7	20.3	14.9	14.6					26.9				26.0			21.8
S. suis type 2	OF	23.4	23.2	21.8	23.3	23.6	22.3	21.3	21.8	19.7	21.6	22.0	21.4			21.2	21.9	23.3	23.8
B. bronchiseptica	OF				25.7							26.6	25.8			25.6			
PCV3	OF			26.6	23.7	19.5	26.8			20.4	22.8					22.3	27.6	29.7	28.8
PPV	OF	26.2	27.4		25.76	18.7	17.9	28.4	30.3			27.1	30.7				32.3		
M. hyopneumoniae	OF																		
M. hyorhinis	OF	26.3	25.9	26.3		23.9		25.8	23.2	21.4	23.2	24.7	24.5			22.0	24.9	24.7	
P. multocida	OF							25.4		19.4							34.8		
B. pilosicoli	FS															21.6			
L. intracellularis	FS			21.6	20.5	20.7	16.8			24.9	26.2	22.4	15.4			12.1	10.2	19.0	18.8
E. coli F4	FS	20.7	24.1					21.9	22.0					14.6	15.3				
E. coli F18	FS	22.8						24.0	26.6	24.2									
PCV3	FS					27.9								24.9	23.1				
PCV2	FS					15.7	16.2												
Rotavirus A	FS	26.1	19.1	30.5	32.9	29.7		22.9	21.2	21.0	17.9	29.7		13.5	13.1	24.2	23.2	21.0	15.7

D) 3N – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		10		24		38		12		26		40		16		30		45	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF	13.0	24.0			26.1		13.5	14.9							22.5	13.2	11.7	21.4
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	14.2	14.0	17.5	11.3	16.7	20.1	15.0	11.5	12.2	14.4	18.2	17.3	11.4	10.9	10.8	14.0	20.2	21.4
A. pleuropneumoniae	OF																		
PCV2	OF													23.4		25.3			
S. suis type 2	OF	20.0	24.1	24.2	22.3	24.3	22.6	18.9	19.4	21.4	22.6	20.7	19.5	21.8	22.6	22.4	24.5	21.8	22.3
B. bronchiseptica	OF	23.1				26.3		25.6	28.5	27.5		26.3		28.1			27.7		
PCV3	OF	16.4	26.8	26.7	27.4	25.9		28.9	19.6	27.6	21.9	21.9	26.4		26.2	25.8		24.2	25.0
PPV	OF																		
M. hyopneumoniae	OF																		
M. hyorhinis	OF			25.6	21.8	23.7	22.9	25.2				22.6	21.1	17.9	20.9	22.0	23.0	24.9	21.1
P. multocida	OF							24.9	26.6	27.9	30.0								
B. pilosicoli	FS								21.6	17.2	23.3		26.5			18.6	19.6		19.3
L. intracellularis	FS					23.6					21.4	16.9		21.1		26.8		15.8	15.3
E. coli F4	FS	22.4											17.2					25.4	
E. coli F18	FS	15.9	19.8		24.8				25.3	20.9			21.2	22.1		27.7			18.9
PCV3	FS	24.0	24.3						25.3			22.3							
PCV2	FS		25.7																
Rotavirus A	FS	24.0	22.9		25.4			19.4	21.0	26.4	23.7			19.4	23.0	25.7			

E) 3F – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		15		35		70		14		44		64		16		46		70	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF		25.7		24.2			18.3						22.8					
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	22.8	20.8	19.0	22.5			20.3	17.0		21.9	20.2	21.6	23.7	22.8	20.4		21.0	
A. pleuropneumoniae	OF																		
PCV2	OF	20.2						21.8	26.0		16.8			19.7	23.1		28.5		
S. suis type 2	OF	22.4	21.9	21.6	20.4	23.9	25.1	21.2	21.9	23.4	23.9	21.5	21.3	24.2	25.8	27.7	23.3	21.4	24.3
B. bronchiseptica	OF							26.0						26.7					
PCV3	OF	16.5	18.5	26.4	20.0	24.9		25.2		29.0	22.8	17.0	24.4	30.8	28.8		28.5	21.2	
PPV	OF	24.5	27.0					27.1						30.4	27.2				
M. hyopneumoniae	OF																		
M. hyorhinis	OF	24.5	23.6	25.7	25.1			20.2	21.6		27.9		25.2	20.6	22.9			26.0	
P. multocida	OF																		
B. pilosicoli	FS			21.4		20.0	15.9		25.0			23.6	23.4			17.5	22.0		20.5
L. intracellularis	FS	17.6			25.6			23.2	18.1						18.1	26.7			
E. coli F4	FS																		
E. coli F18	FS		19.2												20.6	31.0			
PCV3	FS	25.2	24.9	27.7	23.9	29.1	27.9			29.5	28.6	27.2							
PCV2	FS						25.5	27.9						24.2		27.5	27.5		
Rotavirus A	FS		22.4	29.4			24.9		20.7					26.0	31.6				

F) 4N – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		17		31		45		17		31		45		20		34		48	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF				25.5														
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	13.2	17.8	15.0	11.5	18.3	17.6	19.8	17.1	13.3	12.4	15.6	18.2	16.1	16.0	16.3	13.7	20.8	21.0
A. pleuropneumoniae	OF																		
PCV2	OF	28.4	26.2		24.6			24.6	25.8	28.0	24.7		27.6	26.0			33.4	22.8	24.6
S. suis type 2	OF	19.4	22.2	21.2	22.1	20.2	22.0	19.9	20.6	20.8	22.4	20.8	20.0	22.3	22.1	20.6	20.2	21.1	21.8
B. bronchiseptica	OF	25.5	26.9	25.5	24.7	28.1	26.3			24.8									28.4
PCV3	OF							27.3		24.7		27.5				24.0		25.9	31.0
PPV	OF																		
M. hyopneumoniae	OF																		
M. hyorhinis	OF	22.5	24.6	25.1				19.6	20.8	23.7	24.5	27.5	25.1		23.4	25.3		23.4	27.1
P. multocida	OF							27.9											
B. pilosicoli	FS				17.7			27.2	24.9		25.0	17.5	17.6			18.1	14.7	16.0	15.5
L. intracellularis	FS						25.2											16.9	21.4
E. coli F4	FS	23.8	25.6	19.9	23.6			21.9						17.3	17.2	27.4			
E. coli F18	FS	14.1	18.3					14.1	22.0	18.3				16.4	14.3				
PCV3	FS	26.5																	
PCV2	FS	27.2						26.7			28.3								
Rotavirus A	FS	24.5	22.5	27.4	31.2	25.4	26.3	28.4	25.4	23.9				31.4					

G) 4F – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		10		38		74		24		38		66		13		24		69	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF						24.4												
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	17.0	18.4	22.3	21.6			22.0	19.3	26.4		23.7	23.2	19.8	18.3	20.4	22.0	23.6	20.1
A. pleuropneumoniae	OF																		
PCV2	OF	22.4	23.7	6.9	9.3	15.5	15.8	11.7	12.7	10.4	27.7	15.9	13.6	12.7	28.0	21.7		14.9	14.6
S. suis type 2	OF	22.3	22.0	23.7	23.4	23.5	21.5	29.5	24.8	24.4		21.8	21.5	20.4	22.1	23.0	24.8	25.4	20.9
B. bronchiseptica	OF													25.7	27.6				
PCV3	OF				24.7		25.5			19.6			23.0	26.9					
PPV	OF	27.3	28.0		27.7			29.1		16.8	23.5	23.3	25.0					21.2	25.9
M. hyopneumoniae	OF																		
M. hyorhinis	OF	27.9	26.8						25.7	26.4		28.6		24.4					
P. multocida	OF	25.7	23.7																
B. pilosicoli	FS		24.0	19.6	21.7		18.9			18.6	18.4	18.5	19.7	25.1	23.9			21.1	25.4
L. intracellularis	FS	24.5	16.9	17.9			25.3	18.4	14.3	21.2	26.6	24.0	27.2	15.7	15.0	22.2	16.9		
E. coli F4	FS												26.6						
E. coli F18	FS																		
PCV3	FS				24.7		25.5			24.8									
PCV2	FS	20.5	25.2	8.1	9.9	18.9	19.8	11.3	15.2	12.1	17.1	16.5	16.2	12.6		29.5		17.4	17.0
Rotavirus A	FS	22.0				27.8	27.2												

H) 5N – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		12		33		46		12		33		46		15		29		43	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF	11.6	10.4		21.0	28.0		17.0	15.8		20.1		25.6	12.1	10.6				
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	18.9	15.3	12.0	12.4	21.8	21.2	14.0	15.9	16.4	13.6	21.0	20.8	11.1	12.2	11.1	12.1	19.0	18.3
A. pleuropneumoniae	OF																		
PCV2	OF			24.9	20.9	21.4	23.2	19.9	11.5		23.8	17.8	19.2	21.5	23.1	17.8	13.6	19.6	15.9
S. suis type 2	OF	20.1	23.2	23.5	22.3	23.9	21.7	20.3	23.3	22.0	21.8	22.5	22.5	19.1	19.2	18.9	21.5	21.5	21.2
B. bronchiseptica	OF				28.6		27.3	28.0	25.4	27.2			25.9	25.9	24.3	24.5		28.2	
PCV3	OF			28.8	22.6	24.9	21.2		28.3		26.3		28.9	17.2		20.8		21.6	16.6
PPV	OF																		
M. hyopneumoniae	OF																		
M. hyorhinis	OF	23.6	25.5	21.9	24.0	27.8	25.5	19.6	21.4	22.8	23.0	27.4	23.3	19.0	18.7	20.4	20.6	21.7	21.8
P. multocida	OF												26.9	28.7	32.3				
B. pilosicoli	FS														20.3				13.4
L. intracellularis	FS					19.7	25.0					11.8	20.5					18.6	25.7
E. coli F4	FS			24.7				29.9	23.9					16.7					
E. coli F18	FS	25.2	23.6			27.6			17.4	15.9	29.4			18.9	21.1	26.6			
PCV3	FS													25.8					28.7
PCV2	FS			25.9		25.8	19.8	21.3	12.5			16.1	21.2	24.3	22.6	21.1	16.2	23.2	16.0
Rotavirus A	FS	18.4	22.5		22.4	28.2		19.8	24.0	26.8	24.0	27.5	21.7	19.3	13.0	27.7	24.3	24.8	23.1

I) 6N – oral fluid (OF) and faecal sock (FS) samples																					
Sampling date		September						October						November							
Days after insertion		17		31		45		17		31		52		15		29		50			
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2		
swIAV	OF		22.1	14.7		14.1			18.4				27.2		22.9	15.9					
H1 (A(H1N1)pdm09)	OF					25.2															
PRRSV type 1	OF																				
PRRSV type 2	OF																				
PCMV	OF	10.8	16.2	19.0		21.8	19.6	14.7	10.7	14.6	11.3	20.7	23.0	11.0	12.8	12.7	13.2		18.4		
A. pleuropneumoniae	OF																24.9				
PCV2	OF	21.9	21.8	16.4		8.5	18.1	22.1	20.6	22.1	22.7	15.8	9.1	19.7	20.2	24.2	23.0	15.9	15.5		
S. suis type 2	OF	25.4	24.5	22.7		22.5	22.0	23.8	23.3	22.8	22.3	20.4	22.2	19.8	21.5	21.7	22.0	22.7	21.8		
B. bronchiseptica	OF						26.1			25.8		26.2		27.8	27.7	26.2					
PCV3	OF	29.3		26.5			28.2		27.4			27.0		25.0		24.9	27.7	27.6			
PPV	OF					24.1															
M. hyopneumoniae	OF																				
M. hyorhinis	OF																				
P. multocida	OF							27.4		27.3		30.1		24.4		26.2					
B. pilosicoli	FS			25.1	23.1	20.0				25.8	21.5	15.1	14.7				20.8	16.2	21.2		
L. intracellularis	FS			17.1	18.6	18.6	24.3		14.6		21.2	13.7	19.7			24.9	19.9	14.4	15.3		
E. coli F4	FS													20.4	21.8	27.3					
E. coli F18	FS	20.8	23.3					22.0	25.1					19.9	22.1						
PCV3	FS																30.3				
PCV2	FS			17.2	13.1	13.6	19.5	26.7	20.1			18.8	11.6					19.0	20.3		
Rotavirus A	FS	28.6	26.0	24.1	30.1			16.0	23.6	20.6	24.9			18.7	17.3	23.7		22.5			

J) 6F – oral fluid (OF) and faecal sock (FS) samples																			
Sampling date		September						October						November					
Days after insertion		14		30		56		14		42		62		19		40		68	
Pathogen	Material	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2	Pen 1	Pen 2
swIAV	OF							21.5						21.7	26.9				
H1 (A(H1N1)pdm09)	OF																		
PRRSV type 1	OF																		
PRRSV type 2	OF																		
PCMV	OF	21.4	23.1	26.3	24.1			23.3	21.3	21.3	22.1	24.1	21.5	21.4	23.1	21.0	23.3	25.6	26.9
A. pleuropneumoniae	OF	25.4	26.9	25.5	24.1	24.6	25.6			23.7		24.5	23.0		24.9			25.4	22.9
PCV2	OF	13.7	14.4	14.4	24.4	19.5	20.3	11.5	11.9	12.6	19.0	16.4	15.6	9.0	8.5	18.5	18.0	17.4	13.0
S. suis type 2	OF	26.1	24.6	21.1	22.7	23.6	23.9	22.4	22.0	23.0	23.6	20.7	19.9	21.0	21.1	24.8	28.6	21.1	23.0
B. bronchiseptica	OF																		
PCV3	OF		28.1	26.9			30.6		23.7	17.1				24.9	24.0	25.9		27.0	25.5
PPV	OF	21.2	14.0	23.8		22.8	24.4	20.2	23.6	18.3	26.5	19.2	26.2	13.6	19.5	20.0	22.2	22.8	22.4
M. hyopneumoniae	OF										22.6		24.5						
M. hyorhinis	OF																		
P. multocida	OF											28.0	28.0						
B. pilosicoli	FS	19.3	20.3	21.2	22.9	19.2	16.7	15.4	17.9	19.0	18.3	17.6	19.6	22.5	16.9	16.8	18.6	17.0	24.0
L. intracellularis	FS		17.1					16.4						19.3	19.5				
E. coli F4	FS	28.0		28.9	27.6														
E. coli F18	FS									25.2				28.9		28.2			
PCV3	FS								25.4	23.7	27.8								
PCV2	FS	16.6	16.8	15.9	17.1	20.8	21.3	13.9	12.8	16.0	20.0	19.6	17.8	10.7	10.2	19.8	20.0	22.9	18.8
Rotavirus A	FS								25.8	29.3	26.8					33.5	26.7	29.0	

4 Discussion and Perspectives

Respiratory and intestinal diseases are of major importance in the pig industry as they are known to result in production losses due to decreased growth, increased mortality and increased use of medication. Respiratory and intestinal diseases in pigs can be caused by a vast number of viruses, bacteria and parasites or by the toxins they produce. In some cases, a single pathogen is the cause of disease while in others it can be a polymicrobial problem caused by infection with one or more pathogens. Furthermore, the diseases can be the result of a multifunctional problem including pathogen(s) and e.g. stress factors like environmental, nutritional and psychological. Pigs are often housed in large groups and in small spaces, which provides ideal conditions for maintaining circulating pathogens. In addition, the continuous flow of new animals lowers the pressure on the existing pathogens and allows them to thrive. Pathogens involved in respiratory or intestinal diseases in pigs can vary significantly between herds, production sites, regions and countries, which makes standardised treatment and control difficult (Heo et al., 2013; Opriessnig et al., 2011). Therefore, to keep up with the pathogens and their evolution, accurate diagnostic tools, which makes is possible to detect the presence of the infectious agent, is of the outmost importance.

Today, the trend in veterinary diagnostics is moving towards the application of rapid methods, which can provide definitive answers within 24 hours. These rapid methods should be sensitive and specific and be provided at a low cost (Deb and Chakraborty, 2012). Low cost is of high importance to veterinary diagnostics, in that the sampling and subsequent laboratory analyses can become an expensive affair for the farmer, especially in cases where a high number of samples have to be analysed. The high costs caused by either expensive analysis methods or due to a high number of samples can lead to limitations in sampling and analysis. On the contrary, the lack of laboratory investigations can result in overuse of medication such as antibiotics and can lead to suboptimal vaccination programs, since the treatment is based on clinical manifestations instead of actual laboratory test results. Therefore, this PhD aimed to develop a new method for veterinary diagnostics in which the simultaneous detection of multiple targets, a high sample throughput, high sensitivity and specificity were desired. To date, different tools exist for simultaneous detection of multiple pathogens, including microarray hybridization, multiplex PCR and sequencing (Mcloughlin, 2011). However, an ideal diagnostic system should also possess the ability to handle a high number of samples and, therefore, the high-throughput qPCR platform BioMark (Fluidigm) was selected for our diagnostic purposes as it allows for simultaneously analysis of multiple samples in several assays. This platform has already been applied in several studies investigating

expression of immune genes in relation to the presence of pathogens (Skovgaard et al., 2013). More recently, the BioMark platform has also been used as surveillance and detection tools for tick-,food- and waterborne pathogens (Ishii et al., 2013; Michelet et al., 2014; Spurgeon et al., 2008). However, to our knowledge, the high-throughput qPCR platform has not yet been used as a diagnostic tool for veterinary pathogens. In the present PhD project, the high-throughput qPCR platform was utilized for subtyping of swIAVs. In addition, a highly versatile system was established for screening and detection of significant porcine respiratory and enteric pathogens.

In this PhD project, swIAV has been one of the pathogens with most focus due to its importance in the Danish pig production. Since 2011, a systematic passive surveillance of swIAVs has been conducted in Denmark, which has contributed to a detailed knowledge of the circulating subtypes in Danish pigs. Furthermore, surveillance of swIAVs is important due to their zoonotic potential and high evolution rate, which can lead to new emerging virus variants (Kuntz-Simon and Madec, 2009). New reassorted swIAVs have also been discovered during the surveillance in Denmark (Breum et al., 2013; Krog et al., 2017). Since pigs can be infected by a multitude of different variants of HA and NA, even within a subtype, several PCR assays are necessary to cover all the variants, and for conducting adequate analyses. The analysis is always a question of time and cost and in order to minimize these, the use of a high-throughput qPCR system was explored for subtyping of swIAVs. The high-throughput PCR-based analysis contain RT-qPCR assays targeting the different lineages of H1, H3, N1 and N2 circulating in pigs in Europe, together with assays specific for the internal genes of A(H1N1)pdm09. By including the pandemic internal genes, a more detailed subtyping was obtained compared to more traditionally subtyping (Bonin et al., 2018; Henritzi et al., 2016). However, to obtain even more comprehensive subtyping, RT-qPCR assays targeting the non A(H1N1)pdm09 internal genes should be included. In order to detect all the circulating subtypes, it is important to carry out continuous sequencing of selected isolates. This is necessary to maintain the applicability of the PCR-based assays, since sequence changes occur over time due to the high mutation- and reassortant rate of IAVs.

In addition to swIAV, many respiratory, of which several of these are included in PRDC, and enteric pathogens also cause problems in modern pig production. To keep track on these pathogens, it is important to have sensitive and specific diagnostic analyses. Each year, the diagnostic department of the Danish National Veterinary Institute receives thousands of samples submitted for analysis of specific pathogen(s) selected based on the veterinarian´s assessment of the sampled pigs.

In return, the diagnostic department provides different analysis packages, allowing for detection of three to five pathogens. Furthermore, additional analyses can be chosen for an extra cost. The analyses are performed as single qPCR tests, making the diagnostics both costly and time demanding especially if the veterinarian or farmer wants the submitted samples to be tested for several pathogens. The use of the high-throughput qPCR platform instead of the current low-throughput qPCR platform for the pathogen detection enables the analysis to be performed in one test, which reduces the analysis costs and working time substantially. Furthermore, the possibility of including 24, 48 or 96 different qPCR assays, depending of the choice of DA chip, allows for the obtaining of a lot of information. To take advantage of this opportunity, a high-throughput diagnostic system specific for significant respiratory and enteric viral and bacterial pathogens with impact in the Danish pig production was established. For this work, the qPCR assays were designed with specificity towards viruses and bacteria, however, to obtain a more adequate analysis the inclusion of assays specific for parasites, which are also considered pathogenic in pigs, will be necessary. Different types of parasites have been detected in Danish pigs (Joachim et al., 2004; Roepstorff et al., 1998; Roepstorff and Jorsal, 1989). Furthermore, a study has found that *Ascaris suum* significantly compromise the effect of *M. hyopneumoniae* vaccination in pigs (Steenhard et al., 2009). Therefore, qPCR assays specific for different types of parasites should be included. Likewise, a qPCR assay specific *H. parasuis*, which is the causative agent of Glässer's disease (Oliveira and Pijoan, 2004), should be included. Initially, an assay specific for *H. parasuis* was included in this work, however, due to unsatisfactory results when using the BioMark platform, the assay was removed from the analysis. Instead, further optimizations are required for this assay.

We have developed a high-throughput diagnostic system with specificity to seven viruses and ten bacteria and the pathogens, which are included in the analysis packages provided by the diagnostic department, are among these 17 pathogens. The high-throughput diagnostic system was used for objective health monitoring of nursery and finisher pigs in ten pig herds in Denmark. Three monthly samplings were conducted in three different age groups in six nursery and four finisher herds. The laboratory findings combined with clinical sign observations provided information about the pathogen patterns and showed if there was a connection between the presence of pathogens and clinical signs. This information can be useful for veterinary consultancy as it provides the herd veterinarians with information suitable for determining intervention regimes such as vaccination and medication like antibiotic treatment. The veterinarian participating in the project was interviewed and indeed confirmed that the results provided useful knowledge for their consultancy.

In general, antibiotics are giving to treat or to prevent infections, however, in some countries it is also used to increase growth of the animals. Antibiotic consumption in pig production has long been on the political and public agenda in Denmark due to the rise in antibiotic-resistant bacteria. During the last decades, various measures, including preventing veterinarians from profiting from selling antibiotics to farmers and stopping the use of antibiotics as growth promoters, have been taken to reduce the consumption of antibiotics in Denmark (Aarestrup, 2012). Furthermore, to reduce the antibiotic usage even more sensitive and specific laboratory analyses are important to clarify which pathogens are present in the affected herds. Therefore, the combination of high-throughput detection and continuous monitoring provides veterinarians with a high information load, and thereby forms a good basis for choosing the most optimal treatment. Furthermore, a very detailed diagnostic documentation can also be used by the farmer as a scientific argumentation towards the authorities in cases where the farmer needs to use much antibiotics and end up in exceeding the "yellow card" line.

The distribution of pathogens can change over time and often vary across herds, regions and countries (Heo et al., 2013; Opriessnig et al., 2011). Therefore, it is important to adjust the analyses according to the different needs. A major advantage of the high-throughput platform is the ease of modifying the assay panel through the adding or removing of assays. However, these types of qPCR-based assays only provide information about the pathogens which the qPCR test is specific for. For some of the sampled pens (manuscript III), in which diarrhoeic events were observed, none of the intestinal pathogens included in the qPCR analysis were detected. This could indicate that the diarrhoea was caused by another pathogen or as a result of environment or management problems. However, this problem could also arise if a pathogen has mutated in the gene, which the qPCR test is specific for. When observing symptoms like coughing and diarrhoea without any supportive laboratory results it will be a good idea to optimize the test analysis by looking for other relevant pathogens. In this project, no further analysis was done in the herds where pens with diarrhoeic events were observed due to only sporadic findings.

The high-throughput qPCR BioMark platform is a very useful method for analysis of a high number of samples in several different assays. Prior to running the DA chip, all of the assay and sample inlets have to be loaded with assays and samples, respectively, or with e.g. water. As a consequence, this platform is not necessarily beneficial for analysis of only a few samples in a few assays unless the samples and assays have to be run in several replicates. However, different types

of DA chips with variable technical specifications are available. Another aspect to keep in mind when using the high-throughput qPCR platform is the risk of false negative results, which can occur if a sample is very positive. Having a very positive sample on the Rotor-Gene Q platform will result in an even lower Cq value on the BioMark platform due to the additional pre-amplification. Samples with low Cq values will appear light yellow in the heat map, however, the amplification curve might not have the right shape, which can question the result. Therefore, in these cases the sample should be diluted and re-tested on the BioMark platform. To ensure that the qPCR assays were running correctly positive controls for each of the assay were included. When using the diagnostic system in which different types of pathogens were targeted, mixes containing several of the positive controls were used to minimize the number of inlets used for positive controls. For the diagnostic system, the mixes contained up to 13 different positive controls and were successfully included in each run. Negative controls are also necessary to include to control for contamination, both in the cDNA/pre-amplification run and in the qPCR analysis. Furthermore, to determine if factors present in the samples could inhibit the PCR analysis the inclusion of an internal control is preferred (Rosenstraus et al., 1998). Another important aspect is to verify if the assays and samples have been loaded correctly in the reaction chambers on the DA chip. This can be done in the Fluidigm qPCR analysis software by monitoring the ROX data.

Several pathogens are commensal inhabits of the respiratory system or intestinal tract and, therefore, positive PCR findings does not necessarily equal disease. Therefore, correlation between disease and excretion load for a specific pathogen may be expected. Using qPCR enables determination of the pathogen load in e.g. faeces, and this measurement can be useful to confirm if a pathogen is the microbiological cause of disease or not (Pedersen et al., 2012). Correlation between pathogen load and disease severity has been shown to be important for several of the intestinal pathogens, since different loads have been associated with specific clinical diseases (Grau-Roma et al., 2011; Pedersen et al., 2013; Weber et al., 2017). In the present PhD project, the obtained qPCR results have only been given as Cq values for all the included pathogens. However, for PCV2, *L. intracellularis, B. pilosicoli, E. coli* F4 and *E. coli* F18 the results need to be quantitative. Therefore, to improve the high-throughput diagnostic system even further the assays should allow for also quantitative analysis of the investigated pathogens. To do so, standard curves generated by spiking suspensions of the different pathogens in 10% faeces in ten-fold dilutions should be made prior to DNA extraction and run on the high-throughput qPCR platform (Ståhl et al., 2011).

In the present PhD project, the focus has been on designing a diagnostic system specific for porcine pathogens. However, the high-throughput qPCR platform could also be useful as a detection or screening tool for pathogens causing disease in other animals or in humans. Like subtyping of swIAVs has been conducted using the high-throughput qPCR platform, a corresponding system used for subtyping of avian influenza viruses is on the way. This system will be specific for the currently detected HA and NA subtypes (Fouchier et al., 2005). Furthermore, a diagnostic system specific for bovine pathogens is also being developed and here viruses and bacteria often found in cattle are included. So far, qPCR assays specific for *Mycoplasma bovis*, *Mannheimia haemolytica*, *Histophilus somni*, bovine respiratory syncytial virus, bovine parainfluenza type 3 virus and bovine coronavirus have been designed, however, more assays will be added. Similar system could be developed for other important production animals such as poultry and mink or companion animals such as cats, dogs and horses. Another interesting application of the system could be to include qPCR assays specific for resistant genes, which could provide additional layer of information to the analysis especially in relation to antibiotic treatment.

5 Conclusion

The aim of the present PhD project was to establish a cost effective, fast and high-throughput laboratory diagnostic system with high sensitivity and specificity to detect pathogens with high importance to the Danish pig production. In order to fulfil this aim, the previous low-throughput Rotor-Gene Q qPCR platform was replaced with the high-throughput qPCR BioMark platform, which allows for the simultaneous detection of different pathogens in a large number of samples.

The high-throughput system used for subtyping of swIAVs was developed and verified by screening virus isolates and field samples. The use of this system for subtyping enabled the determination of both HA and NA genes and to clarify if the virus contains pandemic internal genes in the same test. Furthermore, a high-throughput diagnostic system was developed for detection of significant respiratory and enteric viral and bacterial pathogens with importance to the health and welfare of Danish pigs. The diagnostic system was designed with specificity to 17 selected viruses and bacteria. The use of this system for monthly diagnostic monitoring of pathogens in ten Danish pig herds showed that the system can be a supportive tool in veterinary consultancy combined with clinical observations, which creates a more objective basis for intervention like treatment and vaccination strategy.

When the diagnostic system is fully implemented and automatized the analysis cost per sample will be less than 5% of the cost of traditional qPCR (own calculations) and thereby this system has great potential for future disease surveillance of pathogens in production herds. The hope is that the lowered costs and time spent on analysis will result in a significant increase in the use of laboratory diagnoses, which in turn should provide an objective basis for selection of preventive measures to the benefit of animal health, animal welfare, production economics and food safety.

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